REMARKS

STATUS OF THE CLAIMS:

Claims 1 to 40, 48 to 49, 51, 58 to 63, and 66 are cancelled.

Claims 41, 52, 53, and 65 have been amended.

New Claims 67 to 72 have been added.

Claims 41 to 47, 50, 52 to 57, 64 to 65, and 67 to 72 are pending.

Claim 41 has been amended to delete subpart "e" in its entirety, and to append the term "and" after subpart "c" to place this claim in proper Markush form in consideration of the deletion of subpart "e". Applicants right to equivalents of Claim 41 is reserved. These amendments were made solely to facilitate prosecution and was not made to overcome any issues related to the patentability of this claim. Applicants right to equivalents of Claim 41 is reserved. No new matter has been added.

Claim 41 has been further amended to substitute the incorrectly spelled term "complimentary" within subpart "d", with the correctly spelled term "complementary" to address the Examiner objection to the same, in addition to delete the phrase "(antisense)" to place this claim in better condition for allowance. These amendments were not made to overcome any issues related to the patentability of this claim. Applicants right to equivalents of Claim 41 is reserved. No new matter has been added.

Claim 52 has been amended to delete the phrase ", and (e)", and to append the term " and" after "(b)," in order to reflect the deletion of subpart "e" from Claim 41. This amendment was made solely to correct the antecedent basis of this claim and was not made to overcome any issues related to the patentability of this claim. No new matter has been added.

Claim 52 has been further amended to replace the phrase "comprising the isolated nucleic acid molecule of a member of the group consisting" with the phrase "comprising a member of the group consisting of the isolated nucleic acid molecule" to address the Examiner's rejection to the same. This amendment was not made to overcome any issues related to the patentability of this claim. Applicants right to equivalents of Claim 52 is reserved. No new matter has been added.

Claim 53 has been amended to replace the plural term "sequences" with the singular term "sequence" to address the Examiner's rejection to the same. This amendment was not made to overcome any issues related to the patentability of this claim. Applicants right to equivalents of Claim 53 is reserved. No new matter has been added.

Claim 65 has been amended to replace the plural term "sequences" with the singular term "sequence" to place this claim in better condition for allowance. This amendment was not made to overcome any issues related to the patentability of this claim. Applicants right to equivalents of Claim 53 is reserved. No new matter has been added.

Support for newly added Claims 67 and 68 may be found in Figure 12, Example 5, Figures 3A-C, SEQ ID NO:5 and 6, and throughout the specification as originally filed. Applicants also refer the Examiner to Exhibit A (submitted concurrently herewith). Exhibit A provides an alignment between the primer pair used to PCR amplify the extracellular region of DmTNFv2 with the encoding nucleotide sequence of DmTNFv2 (SEQ ID NO:5). The encoded polypeptide sequence of DmTNFv2 is also provided (SEQ ID NO:6). As shown, the primers align with a region corresponding to amino acids 62 to 409 of SEQ ID NO:6, which is encoded by nucleotides 817 to 1860 of SEQ ID NO:5. No new matter has been added.

Support for newly added Claims 69 to 70 may be found in Figure 12, and Example 5. Applicants additionally submit concurrently herewith a copy of the Hollenbaugh D. et al paper (EMBO J Dec;11(12):4313-21, (1992)), in addition to the Nakauchi et al paper (PNAS USA, 82:5126-5130(1985)) which refers to and discloses the coding sequence of the extracellular region of mouse CD8/Lyt2a, respectively, for the convenience of the Examiner. Applicants point out that the extracellular DmTNFv2-mouse CD8/Lyt2a fusion protein was specifically isolated using anti-CD8/Lyt2a antibodies as shown in Figure 12 and described in Example 5. No new matter has been added.

Support for newly added Claims 71 to 72 may be found in the paragraph beginning on page 104, line 3, on pages 54 to 58, and in original Claim 66. No new matter has been added.

I. Miscellaneous

a. Objections to the Claims

The Examiner has objected to Applicants Claims 41 subpart (d) stating that the "word "complimentary" (see parts d and e) is spelled incorrectly."

In response, Applicants have amended Claim 41 subpart (d) to replace the incorrectly spelled "complimentary" term with the correctly spelled term "complementary". Applicants have also cancelled subpart (e) of Claim 41. Applicants believe the Examiner's objection of Claim 41(d) has been overcome in consideration of Applicants amendments, and that the Examiner's objection of Claim 41(e) has been rendered moot in consideration of Applicants deletion of the same.

II. Rejections under 35 U.S.C. § 112, second paragraph

a. The Examiner has rejected Claims 41, 50, 51, 52, 56, 58, 60, 62, 63, and 66 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. More particularly, the Examiner has rejected Claims 41, 58, 62, and 66 "as being vague and indefinite in the recitation of the term "tissue". Art teaches that apoptosis are induced in cells and not in tissues (see also page 156, line 25- of the specification). Claims 42-47 50-57, 59-61 and 63-65 are rejected insofar as they depends claim 41."

Applicants disagree. However, in the interest of facilitating prosecution, Applicants have deleted Claim 41 subpart (e), cancelled Claim 58, and amended Claims 62 and 66 to delete the phrase " or tissue". Applicants believe the Examiner's rejection of Claims 41, 58, 62, and 66 have been rendered moot in light of these amendments. In addition, Applicants believe the Examiner's rejection of Claims 42-47 50-57, 59-61 and 63-65 has also been overcome in consideration of Applicants amendments since these Claims 42-47 50-57, 59-61 and 63-65 depend from Claims 41, 58, 62, and/or 66 either directly or indirectly.

b. The Examiner has rejected Claims 41, 50, 51, 52, 56, 58, 60, 62, 63, and 66 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. More particularly, the Examiner has rejected Claim 52 "as being vague and indefinite in the recitation of the phrase ".....vector comprising the isolated nucleic acid molecule of a member of the group consisting of claim....". It is

suggested that Applicants re write the as follows: ".....vector comprising a member of the group consisting of the isolated nucleic acid molecule of claim...".

Applicants disagree. However, in the interest of facilitating prosecution, Applicants have amended Claim 52 to specifically adopt the Examiner's recommended language. Applicants believe the Examiner's rejection has been rendered moot in light of this amendment. Applicants reserve the right to prosecute this claim in its original form in related applications.

c. The Examiner has rejected Claims 41, 50, 51, 52, 56, 58, 60, 62, 63, and 66 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. More particularly, the Examiner has rejected Claim 53 "as being vague and indefinite in the recitation of the term "sequences". A recombinant host cell typically comprises a single vector sequence for expression purposes. Claiming a single vector sequence can obviate the rejection. Claim 54 is rejected insofar it they depends claim 53".

In response, Applicants have amended Claim 53 to substitute the plural "sequences" term with the singular "sequence" term. Applicants believe the Examiner's rejection of Claim 53 has been overcome in consideration of this amendment. Since Claim 54 depends from Claim 53, Applicants believe the Examiner's rejection of Claim 54 has also been overcome.

d. The Examiner has rejected Claims 41, 50, 51, 52, 56, 58, 60, 62, 63, and 66 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. More particularly, the Examiner has rejected Claim 62 "as being vague and indefinite in the recitation of the term "deletions". It is unclear if Applicants are contemplating a single deletion or multiple deletions."

Applicants disagree and point out that the phrase "one or more amino acid deletions" is explicit in indicating that the "deletion" term is meant to encompass one or more amino acid deletions. However, in the sole interest of facilitating prosecution, Applicants have cancelled Claim 62. Applicants believe the Examiner's rejection of Claim 62 has been rendered moot in consideration of this amendment.

Applicants point out that Claim 66 also contains the same "one or more amino acid deletions" language. In an effort to obviate the Examiner's rejection of this claim in a future Office Action, Applicants have canceled Claim 66, and replaced it with new Claim 71. The language of

new Claim 71 has been amended to provide increased clarity in an effort to particularly point out and distinctly claim the subject matter which applicant regards as the invention encompassed by original Claim 66. Since new Claim 71 encompasses the invention encompassed by original Claim 66, though with clarified language, Applicants believe new Claim 71 obviates any potential rejection by the Examiner under 35 U.S.C. § 112, second paragraph and is otherwise allowable.

II. Rejections under 35 U.S.C. § 112, first paragraph

a. The Examiner has rejected Claims 41, 58, 61, and 63 under 35 U.S.C. § 112, first paragraph, alleging that the claimed invention was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. More particularly, the Examiner alleges that "The specification discloses nucleotides of SEQ ID No: 5, nucleotide encoding SEQ ID No: 6. This disclosure meets the written description and enablement provisions of 35 USC 112, first paragraph. However, the specification does not disclose any other nucleotides which hybridize to a nucleic acid comprising the nucleotide sequence of SEQ ID No: 5 or fragments of SEQ ID NO: 5 or nucleotides encoding a protein comprising the amino acid of SEQ ID No: 6 or nucleotide encoding polypeptide fragments consisting of SEQ ID NO: 6 and also comprising nucleotides encoding a polypeptide that induces apoptosis in a cell or tissue. The specification also does not disclose any other nucleotides which is at least 80.0% identical to the nucleotide sequence of SEQ ID No: 5 or fragments of SEQ ID NO: 5 or nucleotides encoding a protein comprising the amino acid of SEQ ID No: 6 or nucleotide encoding polypeptide fragments consisting of SEQ ID NO: 6 or sequences containing deletion or substitutions and also comprising nucleotides encoding a polypeptide that induces apoptosis in a cell or tissue. The claims as written, however, encompass various nucleotide sequences which were not originally contemplated and fail to meet the written description provision of 35 USC 112, first paragraph because the written description is not commensurate in scope with the recitation of claims 41,48, 61 and 63. The specification does not provide written support for the genus encompassed by the instant claims."

Applicants disagree and point out that the specification explicitly discloses hundreds of polynucleotides that are capable of hybridizing to the invention encompassed by Claim 41(e) (see pages 28 to 31, 40 to 44, 54 to 58, and Figure 4 of the instant specification), representative numbers of polynucleotides that are at least "80.0% identical" to the invention encompassed by Claim 58 (see pages 28 to 31, 40 to 44, 54 to 58, and Figure 4 of the instant specification), and explicitly discloses nucleotides encoding polypeptides with one or more amino acid substitutions corresponding to amino acids 316 to 332 of SEQ ID NO:6 (see pages 58 to 60 of the instant specification). However, in the sole interest of facilitating prosecution, Applicants have amended Claim 41 to delete subpart

(e), and cancelled Claims 58 and 63. Applicants believe the Examiner's rejection of Claims 41, 58, and 63 have been rendered moot in consideration of Applicants amendments.

Relative to Claim 61, Applicants believe the Examiner's rejection of this claim under 35 U.S.C. § 112, first paragraph, is in error based upon the fact that the Examiner does not recite nor refer to any of the language from this claim within the body of the rejection under 35 U.S.C. § 112, first paragraph. Rather, the Examiner only recites "nucleotides which hybridize to a nucleic acid comprising the nucleotide sequence of SEQ ID No: 5 or fragments of SEQ ID NO: 5 or nucleotides encoding a protein comprising the amino acid of SEQ ID No: 6 or nucleotide encoding polypeptide fragments consisting of SEQ ID NO: 6 and also comprising nucleotides encoding a polypeptide that induces apoptosis in a cell or tissue. The specification also does not disclose any other nucleotides which is at least 80.0% identical to the nucleotide sequence of SEQ ID No: 5 or fragments of SEQ ID NO: 5 or nucleotides encoding a protein comprising the amino acid of SEQ ID No: 6 or nucleotide encoding polypeptide fragments consisting of SEQ ID NO: 6 or sequences containing deletion or substitutions and also comprising nucleotides encoding a polypeptide that induces apoptosis in a cell or tissue". Accordingly, Applicants request that the Examiner correct the record by withdrawing the rejection of Claim 61 under 35 U.S.C. § 112, first paragraph.

> **b.** The Examiner has rejected Claims 41, 48, 61, and 63 under 35 U.S.C. § 112, first paragraph, alleging that the specification does not reasonably provide enablement for these claims. More particularly, the Examiner alleges "the specification, while enabling for an isolated nucleotide sequence of SEQ ID No: 5, nucleotide sequence encoding SEQ ID No: 6 does not reasonably provide enablement for all possible nucleotide sequences that hybridize to a nucleic acid comprising the nucleotide sequence of SEQ ID No: 5 or fragments of SEQ ID NO: 5 or nucleotide sequence encoding a protein comprising the amino acid of SEQ ID No: 6 or nucleotide sequence encoding polypeptide fragments consisting of SEQ ID NO: 6 and also comprising nucleotides encoding a polypeptide that induces apoptosis in a cell or tissue. The specification is not enabled for any other nucleotide sequence which is at least 80.0% identical to the nucleotide sequence of SEQ ID No: 5 or fragments of SEQ ID NO: 5 or nucleotides encoding a protein comprising the amino acid of SEQ ID No: 6 or nucleotide encoding polypeptide fragments consisting of SEQ ID NO: 6 or sequences containing deletion or substitutions or the various modifications contemplated by the Applicant and also and comprising nucleotides encoding a polypeptide that induces apoptosis in a cell or tissue. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims."

Applicants disagree and point out, as discussed *supra*, that the specification explicitly discloses hundreds of polynucleotides that are capable of hybridizing to the invention encompassed

by Claim 41(e) (see pages 28 to 31, 40 to 44, 54 to 58, and Figure 4 of the instant specification), representative numbers of polynucleotides that are at least "80.0% identical" to the invention encompassed by Claim 58 (see pages 28 to 31, 40 to 44, 54 to 58, and Figure 4 of the instant specification), and explicitly discloses nucleotides encoding polypeptides with one or more amino acid substitutions corresponding to amino acids 316 to 332 of SEQ ID NO:6 (see pages 58 to 60 of the instant specification), and that the skilled artisan could readily make and use the polynucleotides encompassed by these claims based upon techniques readily available in the art of molecular biology as discussed in Applicants prior Reply's. However, in the sole interest of facilitating prosecution, Applicants have amended Claim 41 to delete subpart (e), and cancelled Claim 63. Applicants believe the Examiner's rejection of Claims 41, and 63 have been rendered moot in consideration of Applicants amendments.

Relative to Claim 48, Applicants believe the Examiner's rejection of this claim under 35 U.S.C. § 112, first paragraph, is in error based upon the fact that Claim 48 was a canceled claim at the time the Examiner drafted the instant Office Action. Rather, Applicants believe the Examiner intended to list Claim 58 since he refers to the same language as a basis for his enablement rejection under 35 U.S.C. § 112, first paragraph, as he does in the language used as a basis for his written description rejection under 35 U.S.C. § 112, first paragraph. Applicants request that the Examiner correct the record for clarification. Assuming the Examiner intended to list Claim 58 under this section, Applicants believe the Examiner's rejection of Claim 58 has been rendered moot in consideration of Applicants cancellation of the same.

Relative to Claim 61, Applicants believe the Examiner's rejection of this claim under 35 U.S.C. § 112, first paragraph, is in error based upon the fact that the Examiner does not recite nor refer to any of the language from this claim within the body of the rejection under 35 U.S.C. § 112, first paragraph. Rather, the Examiner only recites "nucleotides which hybridize to a nucleic acid comprising the nucleotide sequence of SEQ ID No: 5 or fragments of SEQ ID NO: 5 or nucleotides encoding a protein comprising the amino acid of SEQ ID No: 6 or nucleotide encoding polypeptide fragments consisting of SEQ ID NO: 6 and also comprising nucleotides encoding a polypeptide that induces apoptosis in a cell or tissue. The specification also does not disclose any other nucleotides which is at least 80.0% identical to the nucleotide sequence of SEQ ID No: 5 or fragments of SEQ ID NO: 5 or nucleotides encoding a protein comprising the amino acid of SEQ ID No: 6 or nucleotide encoding polypeptide fragments consisting of SEQ ID NO: 6 or sequences containing deletion or substitutions and also comprising nucleotides encoding a polypeptide that induces

apoptosis in a cell or tissue". Accordingly, Applicants request that the Examiner correct the record by withdrawing the rejection of Claim 61 under 35 U.S.C. § 112, first paragraph.

Applicants believe that all of the Examiner's rejections and objections have been overcome and that all of the pending claims before the Examiner are in condition for allowance. An early Office Action to that effect is, therefore, earnestly solicited.

If any fee is due in connection herewith not already accounted for, please charge such fee to Deposit Account No. 19-3880 of the undersigned. Furthermore, if any extension of time not already accounted for is required, such extension is hereby petitioned for, and it is requested that any fee due for said extension be charged to the above-stated Deposit Account.

Respectfully submitted,

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Date: January 4, 2005

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Agent for Applicants
Reg. No. 46,652

Exhibit A

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   1  GGCACGAGGCGAACGGACGTTTAAAGTGAGAAAAGAAACCGGTAAATCAGAGATCCCAAG   60

  61  CAAGCGCGTGCGTGCATGATAGCGAAGAAAAAAAGCTATCCGTTTCAGTTAACTACTTAC  120

 121  CAAGATTGAATTTCGCCATCGGGCAAATTACTAAAAATACATAAGTGCAACTCGTCCACT  180

 181  GTGTGTTGTGTTTTTTTTTTTTTTTTGGTTTTCGCTGTGCCTTTATCGCAAACAAGAAC  240

 241  TGATAAAACTAGAAAATATCTTGAGAAACTTGTTTTCGCGCTTTTCTTTTGCTAATTGCC  300

 301  GATCGCGGAAGAGAAAAACAAGCAGTAGACAAAACAAGTGTGGTAATACAATCTGAAAAG  360

 361  GGCACCATCAGCAGCCCGAGGGGTTTATCTATATAGATGTCGCAGCTTATCATCTCATGC  420

 421  TGTCTGTGAGGTTGTTCTGTGTGCTCGTGTAGTATCTTAAATACATAGAGTGTGTTCATA  480

 481  TAAAGTGCGACAAAGCTCGATTGGAAACAGCTGTCGAGTGCCCTTGAGTGGGTGGGCAAG  540

 541  ATCGTCATCATCATCATCGTCGTCATTATCAACAGAATCAGCATCAGCATCTGGAGGCCC  600

 601  CGGTTGCTCTAAGATCCCCAGTGTTCATCAATTATGACTGCCGAGACCCTCAAGCCGTTT  660
   1                                  M  T  A  E  T  L  K  P  F      9

 661  ATAACGCCAACGAGTGCCAACGATGATGGTTTTCCGGCCAAAGCGACCAGCACGGCGACC  720
  10  I  T  P  T  S  A  N  D  D  G  F  P  A  K  A  T  S  T  A  T   29

 721  GCCCAGCGACGCACCCGCCAGCTGATCCCCCTGGTTTTGGGGTTCATCGGTCTGGGGCTG  780
  30  A  Q  R  R  T  R  Q  L  I  P  L  V  L  G  F  I  G  L  G  L   49
                                  DmTNFv2 Primer (SEQ ID NO:21)
                          CGGAAAGATCTAACGCGTGTATCGCATCTGGACAAG
 781  GTCGTTGCCATTCTCGCACTAACGATCTGGCAGACAACGCGTGTATCGCATCTGGACAAG  840
  50  V  V  A  I  L  A  L  T  I  W  Q  T  T  R  V  S  H  L  D  K   69

 841  GAGCTGAAGAGCCTGAAGCGAGTCGTCGATAATCTCCAGCAGCGTTTGGGCATAAACTAT  900
  70  E  L  K  S  L  K  R  V  V  D  N  L  Q  Q  R  L  G  I  N  Y   89

 901  CTGGACGAGTTCGACGAGTTCCAAAAGGAGTACGAGAATGCCCTCATCGACTATCCAAAA  960
  90  L  D  E  F  D  E  F  Q  K  E  Y  E  N  A  L  I  D  Y  P  K   109

 961  AAGGTGGATGGCCTCACGGATGAGGAGGACGACGACGATGGCGATGGTCTGGATTCCATT 1020
 110  K  V  D  G  L  T  D  E  E  D  D  D  D  G  D  G  L  D  S  I   129
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Exhibit A (Cont'd)

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1021  GCGGACGACGAGGACGACGACGTTAGCTATAGCTCTGTGGATGATGTTGGCGCAGACTAC   1080
 130  A  D  D  E  D  D  D  V  S  Y  S  S  V  D  D  V  G  A  D  Y     149

1081  GAGGACTACACCGATATGTTAAATAAACTCAACAATGCACATACCGGCACCACGCCCACA   1140
 150  E  D  Y  T  D  M  L  N  K  L  N  N  A  H  T  G  T  T  P  T     169

1141  TCTGAGACCACTGCTGAGGGCGAGGGCGAGACGGACAGTGCATCCTCAGCCTCAAATGAT   1200
 170  S  E  T  T  A  E  G  E  G  E  T  D  S  A  S  S  A  S  N  D     189

1201  GACAATGTGTTCGATGACTTTACCAGCTACAATGCCCACAAAAAGAAGCAGGAGAGAAAA   1260
 190  D  N  V  F  D  D  F  T  S  Y  N  A  H  K  K  K  Q  E  R  K     209

1261  TCTCGCTCGATTGCCGATGTACGCAATGAGGAGCAGAATATTCAAGGAAATCACACAGAG   1320
 210  S  R  S  I  A  D  V  R  N  E  E  Q  N  I  Q  G  N  H  T  E     229

1321  CTTCAGGAAAAGTCATCCAATGAGGCAACTTCCAAAGAGAGCCCTGCACCACTTCACCAC   1380
 230  L  Q  E  K  S  S  N  E  A  T  S  K  E  S  P  A  P  L  H  H     249

1381  CGTCGCAGAATGCATTCCCGCCATCGCCACCTCCTAGTCCGCAAAGCCAGATCCGAGGAC   1440
 250  R  R  R  M  H  S  R  H  R  H  L  L  V  R  K  A  R  S  E  D     269

1441  TCGAGGCCAGCAGCCCATTTCCACTTGAGCAGCAGGCGGCGTCACCAAGGAAGTATGGGC   1500
 270  S  R  P  A  A  H  F  H  L  S  S  R  R  R  H  Q  G  S  M  G     289

1501  TACCATGGAGATATGTACATAGGAAATGATAACGAGAGAAACTCTTATCAGGGACACTTT   1560
 290  Y  H  G  D  M  Y  I  G  N  D  N  E  R  N  S  Y  Q  G  H  F     309

1561  CAAACGCGCGATGGCGTCTTGACGGTGACCAATACAGGCCTATATTACGTATACGCCCAG   1620
 310  Q  T  R  D  G  V  L  T  V  T  N  T  G  L  Y  Y  V  Y  A  Q     329

1621  ATATGCTACAACAACTCGCACGACCAGAACGGATTTATCGTCTTTCAAGGAGACACTCCA   1680
 330  I  C  Y  N  N  S  H  D  Q  N  G  F  I  V  F  Q  G  D  T  P     349

1681  TTCCTGCAGTGCTTGAACACGGTGCCCACCAACATGCCACATAAGGTGCACACCTGCCAC   1740
 350  F  L  Q  C  L  N  T  V  P  T  N  M  P  H  K  V  H  T  C  H     369

1741  ACGAGTGGTCTGATCCACCTGGAACGAAACGAGAGGATCCATCTGAAGGACATTCACAAC   1800
 370  T  S  G  L  I  H  L  E  R  N  E  R  I  H  L  K  D  I  H  N     389
                                                DmTNFv2 Primer (SEQ ID NO:22)
                                                      GGCATCTTCAAGGTG
1801  GATCGCAATGCAGTTCTGCGGGAGGGAAACAACCGAAGCTACTTTGGCATCTTCAAGGTG   1860
 390  D  R  N  A  V  L  R  E  G  N  N  R  S  Y  F  G  I  F  K  V     409
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Exhibit A (Cont'd)

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      TAAATTTCTAGAGGC
1861  TAAATTGGAGAGATTATCCCCGGTCAGAAGATGGAATACCAGTTTAAGCTTTTGTCCCCG   1920

1921  CGACTGCTCGTGAATGCGATTCATCGCCAGCGTGAATCCATTAGTTCGTAGTACCTAGTC   1980

1981  TTAGTCACTCCAAACCTAATCTCAATCGGAATCGTGCATACTGCATTAGTCAGAAGACGG   2040

2041  AGGAAAATCATATTTATTTTGTATATACTCGTTCGACTCTAAAAAGTGAATAAAAATATA   2100

2101  TGTAGCTATTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACTCGAG  2148
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Immunology

Molecular cloning of Lyt-2, a membrane glycoprotein marking a subset of mouse T lymphocytes: Molecular homology to its human counterpart, Leu-2/T8, and to immunoglobulin variable regions

(cytotoxic/suppressor/differentiation antigen/transfectants/fluorescence-activated cell sorter)

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Contributed by L. A. Herzenberg, April 1, 1985

ABSTRACT **The sequence of Lyt-2 cDNA shows that it is a new member of the immunoglobulin super gene family. Analysis of the predicted amino acid sequence indicates that the Lyt-2 polypeptide is synthesized with a 27-amino acid leader, and that the mature protein has an immunoglobulin variable region (Ig V)-related sequence of ≈100 amino acids, an extracellular spacer of 43, a transmembrane region of 38, and an intracytoplasmic region of 27 amino acids. Lyt-2 and its human analogue Leu-2 are 56% homologous; analysis indicates that the Ig V-related domains of the two molecules have evolved away from each other faster than the carboxyl-terminal half of the proteins.**

Suppressor and cytotoxic T lymphocytes (CTLs) of mice are distinguished by the expression of the lymphocyte differentiation molecule Lyt-2,3 (1, 2). Human T lymphocytes with analogous functions express the molecule Leu-2 (T8, CD8) (3–5). We had postulated that these molecules are not only functionally but also structurally homologous (6). Monoclonal antibodies against the Lyt-2,3 or Leu-2 molecules in the two species block cytotoxic activity of most CTLs that bear them (7–9). This is apparently because Lyt-2,3 and Leu-2 serve an accessory function in the binding of CTL to target major histocompatibility complex class I molecules (9). The role of Lyt-2 (or Leu-2) is indicated to be binding rather than killing, because CTLs bound to target cells with a lectin such as concanavalin A can kill even in the presence of anti-Lyt-2 or anti-Leu-2 antibody (10).

The Lyt-2 antigen is found on 34-kDa and 38-kDa polypeptides disulfide-linked to Lyt-3 primarily as heteromultimers (11). On peripheral T cells and some T lymphomas, a small fraction of Lyt-2 exist also as homodimers (unpublished observations). The Leu-2 antigen is similarly found on a 32-kDa chain, which is disulfide-linked with another polypeptide (CD1 or T6) on thymocytes or with another Leu-2 peptide on peripheral blood lymphocytes to form heterodimers or homodimers (12, 13).

To determine the detailed structure of Leu-2 (and subsequently Lyt-2), we isolated molecular clones of cDNA and genomic DNA coding for Leu-2 (14) by using a subtractive cDNA approach with L-cell transfectants selected with the fluorescence-activated cell sorter (FACS) after DNA-mediated gene transfer (14, 15). In this paper, we describe the isolation of Lyt-2 cDNA and genomic clones, present the predicted amino acid sequence and the general structure of the Lyt-2 polypeptide, and compare the Lyt-2 sequence with that of Leu-2 as described (16).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

METHODS

Transfection and Cell Staining. The thymidine kinase deficient (TK^-) L cells were transfected with 19 μg of BALB/c liver cellular DNA, and 1 μg of pBR322 containing chicken *TK* gene (per 10^6 cells) by a calcium phosphate procedure (17, 18). Some of the dishes received 1 μg of pBR322 or phage DNA containing the putative Lyt-2 genomic clone. After 2 weeks of selection in hypoxanthine/aminopterin/thymidine (HAT) medium, TK^+ L cells were stained with rat monoclonal anti-Lyt-2 antibody (53-6.7) (19). Positive cells were selected by sorting, using FACS as described (15).

Immunoprecipitation and $NaDodSO_4$ Gel Electrophoresis. BALB/c thymocytes or Lyt-2 transformants were labeled with ^{125}I by the lactoperoxidase method. Immunoprecipitation and $NaDodSO_4$ gel electrophoresis were carried out as described by Jones (20).

Cloning of Lyt-2 cDNA and Genomic Gene. A BALB/c thymocyte cDNA library (kindly provided by C. Benoit) was plated on *Escherichia coli* C600/HFL. Low stringency hybridization was in 6× SSPE with 0.1% $NaDodSO_4$/4× SSPE washings at 65°C (1× SSPE = 180 mM NaCl/10 mM $NaPO_4$, pH 7.7/1 mM EDTA). A confirmed Lyt-2 cDNA clone (see *Results*) was then used to pick full-length cDNA clones from a C57BL/6 thymocyte cDNA library in λgt10 cloning vector (kindly provided by H. Gershenfeld and I. Weissman) and a genomic clone from a B10.A liver genomic library in λJ cloning vector (21), with washing done at high stringency (0.1% $NaDodSO_4$/0.1× SSPE).

DNA and RNA Hybridization. DNA was digested and analyzed by the Southern blot technique (22). RNA was prepared from cells or tissue by the guanidium thiocyanate procedure (23) and analyzed by RNA blot hybridization (24).

Sequencing. DNA nucleotide sequence was determined by the method of Sanger *et al.* (25) after subcloning restriction endonuclease fragments into M13 mp18 and mp19 phage vectors.

Computer Analysis. Sequences were analyzed on a VAX 11/780 computer using the programs described by Staden (26, 27). Alignment of cDNA, translation of cDNA, and amino acid alignment of Lyt-2 and Leu-2 sequence was done using the BIONET system (National Institutes of Health Grant No. U41 RR01681-01) and the DB system (28). The Dayhoff Protein sequence bank was searched by the program described by Lipman and Pearson (29).

RESULTS

Establishment of Lyt-2 Transfectants. We found the frequency of Lyt-2 transformants after 2 weeks of HAT selec-

Abbreviations: FACS, fluorescence-activated cell sorter; TK, thymidine kinase; CTL, cytotoxic T lymphocyte; Ig V, immunoglobulin variable region; kb, kilobase(s); bp, base pair(s).

tion to be 10^{-4} to 10^{-3} of the TK^+ cells. Interestingly, as we found with Leu-2 transfection, some of the transfectants had higher amounts of Lyt-2 on the cell surface than others. These transformants, therefore, resembled the amplifying transformants we found in 25–50% of Leu-2 transformants (30). After several more rounds of selection for the most brightly staining cells, we established two cloned Lyt-2 amplified cell lines (S2K9, S2Q9). The mean fluorescence of these cells was >10 times greater than that of the original transfectants. The presence of Lyt-2 molecules on these amplified transformants was confirmed by immunoprecipitation and two-dimensional gel analysis (31). We have not detected Lyt-3 determinants on transformants obtained with either total cellular DNA or cloned Lyt-2 coding DNA fragments.

Screening of a Mouse Thymocyte cDNA Library with a Leu-2 cDNA Probe. We screened a BALB/c mouse thymocyte cDNA library (C. Benoit) using our 1.7-kilobase (kb) Leu-2 cDNA as a probe under conditions of low stringency hybridization. Of 152,000 plaques screened, 11 positive phage clones were isolated. After *Eco*RI digestion, each cDNA insert was purified and labeled with ^{32}P by nick-translation. The probe was hybridized to genomic DNA from L cells, and from the two amplified Lyt-2 cell lines, S2K9 and S2Q9 (whose isolation was described above). The cDNA inserts ranged in size from 300 to 1000 base pairs (bp); four were 650 bp long. Only these four gave very intense bands with the DNA obtained from the two amplified lines and a band of the same size (5.2 kb when digested with *Hin*dIII) at single copy intensity with L-cell DNA (Fig. 1*a*). These 650-bp inserts also hybridized strongly with RNA from the amplified transformants and not with L-cell RNA on an RNA blot (data not shown). These four hybridized with each other and not with any of the remaining cDNA inserts. We designated a pSP65 plasmid with this insert in the RI site pLY2C-1 (Fig. 2*b*).

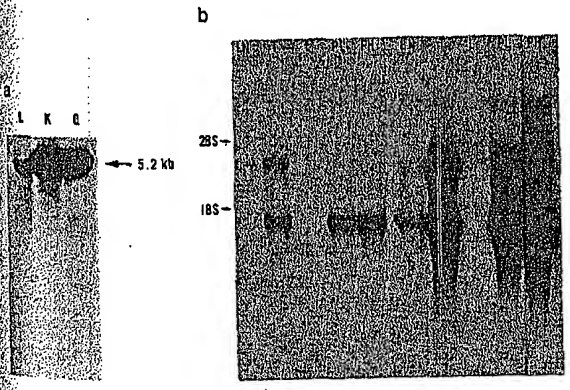

FIG. 1. (*a*) Autoradiogram of Southern blot showing that the Lyt-2 gene is greatly amplified in the genomic DNA obtained from two Lyt-2 amplified transformants. Fifteen micrograms of DNA from L cells (lane L) and 10 μg from the two Lyt-2 amplified lines (lanes K and Q) were digested with *Hin*dIII, electrophoresed through a 0.8% agarose gel, transferred to nitrocellulose filter paper, and hybridized with a ^{32}P-labeled insert of pLY2C-1. (*b*) Autoradiogram of RNA blot from different sources hybridized to a nick-translated Lyt-2 cDNA (pLY2C-1). Fifteen micrograms of total RNA obtained from lymph node cells (lanes LN), thymocytes (lanes T), L cells (lanes L), and two Lyt-2 transfectants (lanes PL and PH), which were transfected with p6CCA plasmid or L-6CCA phage DNA containing Lyt-2 genomic insert, respectively, were loaded in each lane. Two different sized bands are seen in lanes LN and T (1.7 and 3.0 kb), but only the 1.7-kb band is seen in lanes PL and PH. Longer exposure of the same filter (shown on right) allowed visualization of the bands in lane LN as well as multiple distinct bands from 2.5 to 3.8 kb in lanes PL and PH.

Lyt-2 transformants, thymocytes, and lymph node cells revealed two mRNA species, of ≈1.7 and 3.0 kb, which hybridize to nick-translated pLY2C-1. We screened a second thymocyte cDNA library, from a C57BL/6 thymocyte (H.

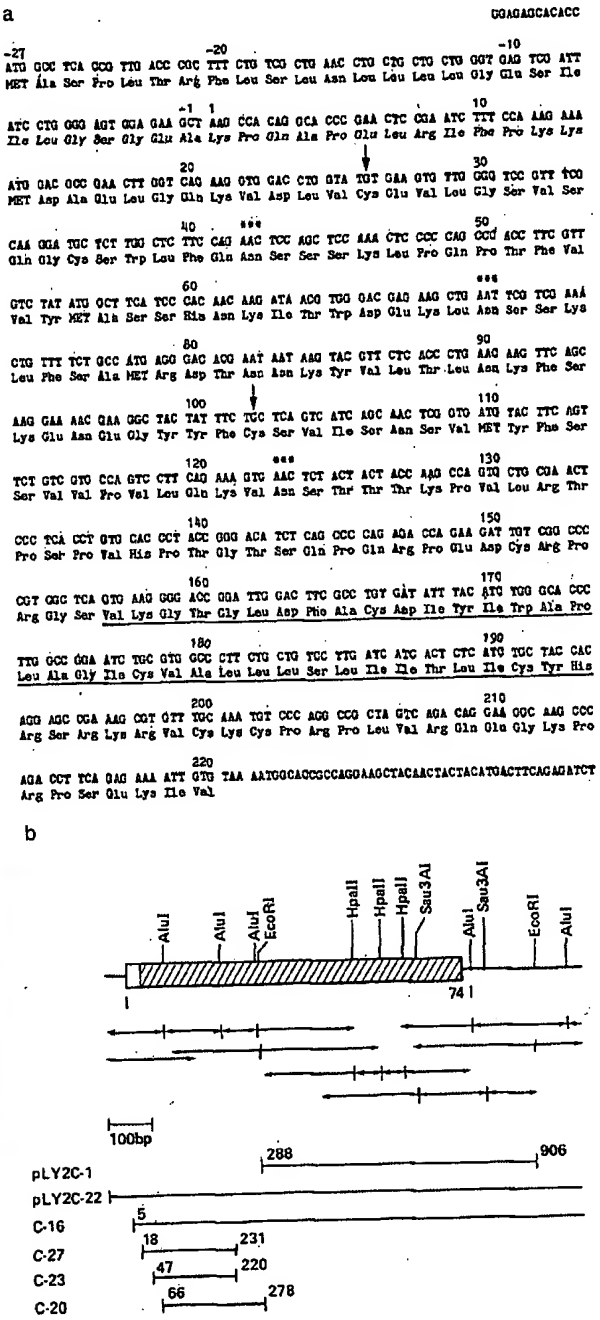

FIG. 2. (*a*) Nucleotide and amino acid sequence derived from thymocyte cDNA clones encoding Lyt-2. Transmembrane region is underlined. Cysteine residues believed to participate in intrachain disulfide bonding are marked by arrows. Possible *N*-linked glycosylation sites are marked by asterisks. The 27 amino acid leader peptide is marked as −27 to −1. Mature peptide is marked from 1 to 220. (*b*) Restriction endonuclease maps of Lyt-2 cDNA clones. Protein coding region is boxed. Shaded region is mature protein after cleavage of leader peptide. DNA sequence strategy is presented below the map. Numbering refers to nucleotide sequence within each clone relative to the ATG of NH_2-terminal methionine.

Gershenfeld and I. Weissman), using pLY2C-1 as a probe, and we obtained the clone pLY2C-22. This clone contains the entire coding sequence plus 12 bp upstream of the ATG start codon.

Since Walker *et al.* reported a major and a minor NH_2-terminal amino acid sequence for Lyt-2 (32), we searched for evidence of more than one Lyt-2 gene. Low stringency Southern hybridization analysis of BALB/c liver genomic DNA using as probe a subclone that lacks the COOH-terminal portion of the protein and 3′ untranslated region of clone pLY2C-16 (bases 992–1393) showed only a single band with *Pst* I and *Hin*dIII digests, consistent with a single gene coding for Lyt-2. Furthermore, we found identical sequences for the NH_2-terminal coding portion of 4 other cDNA clones (C-16, C-27, C-23, C-20) obtained from the C57BL/6 thymocyte cDNA library (Fig. 2*b*).

We screened a B10.A genomic library (21) with the insert of the pLY2C-1 cDNA clone and isolated a λj1 phage (L-6CCA) containing a 15-kb fragment with the Lyt-2 gene. We obtained a subclone in pBR322 (p6CCA) with a 5.2-kb *Hin*dIII fragment, which hybridizes to pLY2C-1. About 25% of TK^+ L cells transfected with either the L-6CCA or p6CCA DNA stained positive for Lyt-2. The presence of the Lyt-2 glycoprotein on cloned transformants was confirmed by immunoprecipitation, showing 38-kDa and 34-kDa polypeptides with multiple charge differences on two-dimensional gel electrophoresis (Fig. 3).

Primary Protein Structure. The nucleotide sequence and predicted amino acid sequence of the Lyt-2 cDNA is presented in Fig. 2*a*. Comparison of the amino acids predicted from the cDNA sequence with the "major" amino acid sequence of purified Lyt-2 protein (32) shows complete correspondence for 16 residues beginning 27 amino acids downstream from the NH_2-terminal methionine (Fig. 2*a*). Hydrophobicity analysis of the protein (data not shown) showed that residues 1–27 have a profile characteristic of hydrophobic leader sequences. These data, taken together with the NH_2-terminal microsequencing data, lead us to conclude that the mature peptide is 220 amino acids (predicted size, 24.7 kDa) and has a leader peptide of 27 residues.

Analysis of the hydrophobicity plot also reveals a region of strong hydrophobicity near the COOH terminus (residues 158–193) followed by a hydrophilic sequence (residues 194–220); these regions we predict to be the transmembrane region and cytoplasmic tail, respectively. Three possible *N*-linked glycosylation sites are present at positions 42, 70, and 123 (Fig. 2*a*). The two different sizes (34 kDa and 38 kDa) of Lyt-2 are reported to be due to differential glycosylation of the same polypeptide sequence (33). Given the observed sizes of Lyt-2 as 34 kDa and 38 kDa, *O*-linked and/or *N*-linked glycosylation must account for 9–14 kDa.

Comparison of Lyt-2 and Leu-2. Comparison of Lyt-2 and Leu-2 at the amino acid level (Fig. 4) shows that they are 56% identical after computer alignment of both sequences. The distribution of homology across the two molecules reveals that the variable-like regions (identities, counting gaps as mismatches, are 42%) of the proteins have diverged more than the COOH termini (identities are 64%).

Most notably, there are nine cysteines in each protein which, after computer alignment of the amino acid sequences, with no bias for alignment of cysteines *per se*, all align with their putative homologue. Given that cysteine residues are important in the formation of inter-/intrachain disulfide bonds, the maintenance of these cysteine residues, and the amino acids immediately surrounding them, indicates their probable functional or structural importance. After computer alignment of the sequences, no Lyt-2 potential *N*-linked glycosylation site is located at exactly the one Leu-2 potential glycosylation site. Comparison of the hydrophobicity plots of Lyt-2 and Leu-2 (data not shown) confirms that the hydrophobic and hydrophilic regions are similar in the two proteins, the major difference being a hydrophilic segment just before the transmembrane region in Lyt-2, which is neutral in Leu-2.

Lyt-2 Is a Member of the Immunoglobulin Super Gene Family. A computer search of the Dayhoff protein sequence bank for sequences similar to Lyt-2 indicates that Lyt-2 is homologous to human and mouse κ light chain variable (V) regions (30% identity, requiring 18 gaps in Lyt-2 and κ sequence to give best alignment), heavy chain V regions (20% identity, requiring 9 gaps); clear but lesser homology was observed with mouse Thy-1 (34), λ light chain V regions, and T-cell receptor α- (35, 36), β- (37), and γ-chain V regions (38).

Alignment of the most significant homologies (Fig. 5) suggests that Lyt-2 has an Ig V-like domain. Residues regarded as important for heavy chain Ig domain structure/function such as the cysteines that form the intrachain disulfide bond as well as an invariant tryptophan are also found appropriately located, after alignment, in Lyt-2 and Leu-2 (Lyt-2 cysteine residues 26 and 102; tryptophan residue 38).

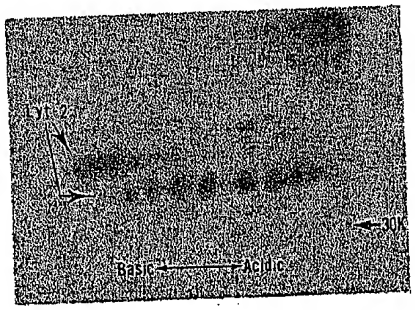

FIG. 3. Two-dimensional gel electrophoresis of the Lyt-2 molecule. A detergent lysate from surface ^{125}I-labeled transfectants generated by using p6CCA DNA was immunoprecipitated with anti-Lyt-2 antibody (53-6.7). The first dimension was a charge separation with the acidic side on the right and the basic side on the left. The second dimension was $NaDodSO_4$/10% polyacrylamide slab gels from the top to the bottom under reducing conditions. 30K, 30 kDa.

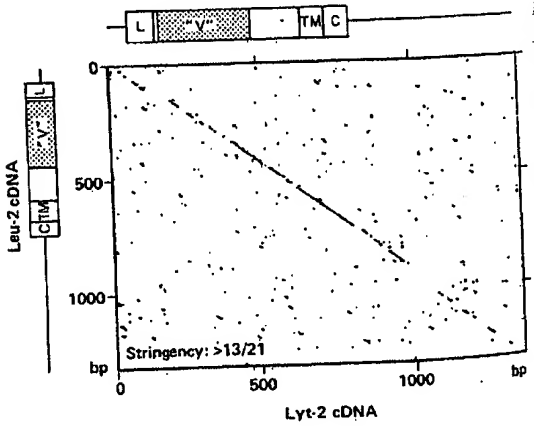

FIG. 4. DNA homology of Lyt-2 and Leu-2. A computer homology representation of the Lyt-2 cDNA sequence versus the Leu-2 cDNA (ref. 16 and unpublished data) using the DIAGON program of Staden (27). Stringency for plotting of homology was (% score = 13)/(odd span length = 21) = 61%. The general structures of the Lyt-2 and Leu-2 proteins in relation to their cDNA coding sequence are presented on the horizontal and vertical axes, respectively.

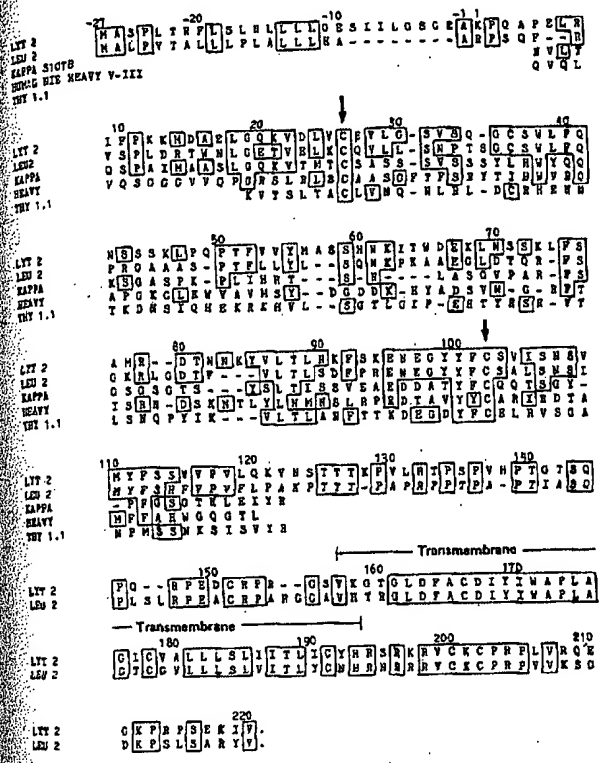

FIG. 5. Lyt-2 homology to other immunologically relevant proteins. Using the BIONET programs IFIND and ALIGN and the protein homology search program of Lipman and Pearson (29), the deduced Lyt-2 protein sequence was aligned against Leu-2 and the most significantly homologous V region sequence of mouse κ light chain, human heavy chain Ig, and the V-like region of Thy-1.1. Homologies of these proteins to Lyt-2 are boxed. The cysteines proposed to make the intrachain disulfide bond to form an Ig-like domain in Lyt-2 and Leu-2 are marked by arrows. The transmembrane region of Lyt-2 is indicated. Amino acids are identified by the single letter code.

Assuming an Ig V-like structure, Lyt-2 resembles a heavy-chain V region (V_H) most in the number of residues between the disulfide-linked cysteines (V_H, 73; V region κ chain, 65; Lyt-2, 75; Leu-2, 71) (39). Because of this observation, we mapped our alignment of Lyt-2 and the Nie Ig V_H region onto a two-dimensional representation of an Ig domain (Fig. 6) (40). Of the 23 residues that are shared between the two proteins, 7 are at positions considered invariant for Ig V regions (39). This can be compared with a total of 14 invariant residues in Ig V-region domains.

DISCUSSION

We have predicted the structure of the Lyt-2 molecule by deduction from DNA sequencing of the cDNA and gene for Lyt-2, and compare it with Leu-2 (T8), which had recently been cloned and sequenced using similar methods (14, 16). We previously suggested the homology of these mouse and human genes from consideration of FACS and $NaDodSO_4$ gel analyses (6). A number of other common properties suggest homology. For example, (*i*) they both are selectively expressed on suppressor/cytotoxic T cells (1–4), (*ii*) monoclonal antibodies against Lyt-2 or Leu-2 block cytotoxicity of most class I but not of class II restricted cytotoxic T cells (7–9), (*iii*) the genes that code for Lyt-2 or Leu-2 are closely linked to the immunoglobulin κ light chain locus in each species (41–43), (*iv*) they are composed of polypeptide subunits that are disulfide-bonded into a variety of multimeric

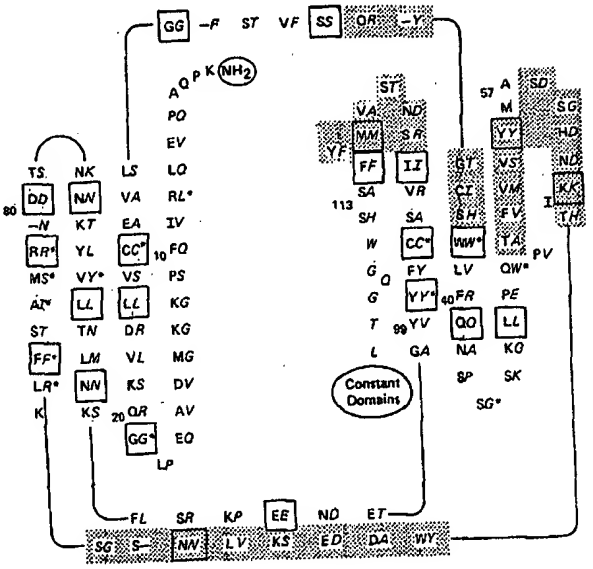

FIG. 6. The Lyt-2 sequence (bold lettering) was mapped, by homology to the human Nie heavy chain V region sequence (italic lettering), onto a two-dimensional representation of the β-pleated sheet structure of the Ig heavy chain V region domain (39). Numbering is from NH_2-terminal of Lyt-2 sequence. Boxed residues indicate homology. Asterisks mark invariant heavy chain Ig V region residues. Shaded regions cover the hypervariable region of heavy chain Ig V domain.

forms (11), (*v*) both genes amplify in a high proportion of transformants after DNA-mediated gene transfection (30, 31).

Here we have shown that by DNA and amino acid sequence comparison, Lyt-2 and Leu-2 are highly homologous to each other and less but clearly homologous with other members of the immunoglobulin super gene family (44). Lyt-2 has a typical leader sequence of 27 amino acids, followed by ≈110 amino acids that show clear homology to Ig V regions. This region also shows homology to Thy-1 (34), OX-2 (45), and T-cell receptor polypeptides (35–38) (data not shown). Thus, these molecules are all related evolutionarily and probably arose from a common ancestral gene. The cDNA sequence of Lyt-2 also codes for a peptide stretch of 43 amino acids COOH-terminal to the region of Ig V homology. The deduced COOH-terminal amino acid sequence has a 38 amino acid hydrophobic stretch, resembling a transmembrane domain, with an abrupt change to a hydrophilic region, probably corresponding to an intracytoplasmic domain of 27 amino acids.

The V region-like domains of Lyt-2 (residues 1–120) and Leu-2 have ≈18% less identity than the COOH-terminal portion of these proteins (Lyt-2 residues 121–220; Fig. 5). Since Lyt-2 and Leu-2 are related to Ig and Ig-like proteins that function in molecular recognition, and Lyt-2/Leu-2 restrict CTL recognition to class I expressing targets, it is interesting to speculate that this recognition of class I is mediated by the Ig-like structures of Lyt-2 and Leu-2. The greater divergence in the V-like regions of these two molecules could be due to their coevolution with their different major histocompatibility complex counterparts: Lyt-2 evolving to recognize H-2 class I, and Leu-2 evolving to recognize class I HLA. Although the finding that Lyt-2 cDNA has an Ig V-like sequence led us to investigate the possibility of DNA rearrangement in this gene, we could not find any evidence supporting this idea.

Comparison of cDNA sequences of Lyt-2 and Leu-2 revealed that the two cDNAs are most similar across those

regions corresponding to coding sequences (Fig. 4). However, the homology also extends into 5′ and 3′ untranslated cDNA, possibly reflecting regulatory sequences that have been evolutionarily maintained.

The origin of two mRNA species remains unknown. It is likely that 1.7-kb mRNA is sufficient for the expression of Lyt-2 molecule since the L-cell transformants receiving 5.2 kb or 15 kb Lyt-2 genomic fragments make abundant Lyt-2 protein even though the amount of 3.0-kb mRNA in these cells is greatly reduced or absent. It should also be noted that both 34-kDa and 38-kDa molecules of Lyt-2 can be immunoprecipitated from those L-cell transformants transfected with either this 5.2-kb (Fig. 3) or 15-kb genomic fragments (data not shown). Therefore, a single gene and the 1.7-kb mRNA seem to be responsible for both the 34-kDa and the 38-kDa glycoproteins; L cells are able to glycosylate Lyt-2 protein similarly to thymocytes.

Preliminary sequence data of the Lyt-2 genomic insert from p6CCA indicate that the Lyt-2 gene is composed of at least five exons. Unlike other Ig-like molecules, Lyt-2 does not have a separate leader exon. The leader peptide, fused to the V-like region, is encoded by one exon. The transmembrane portion is encoded entirely by its own exon; the cytoplasmic region is encoded by two exons.

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The human T cell antigen gp39, a member of the TNF gene family, is a ligand for the CD40 receptor: expression of a soluble form of gp39 with B cell co-stimulatory activity

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Signals delivered to B cells via CD40 can synergize with those provided by other B cell surface receptors to induce B cell proliferation and antibody class switching as well as modulate cytokine production and cell adhesion. Recently, it has been shown that the ligand for CD40 is a cell surface protein of ~39 kDa expressed by activated T cells, gp39. Here we report on the isolation and characterization of a cDNA clone encoding human gp39, a type II membrane protein with homology to TNF, and the construction and characterization of a soluble recombinant form of gp39. COS cell transfectants expressing gp39 synergized with either anti-CD20 mAb or PMA to drive strong B cell proliferation and alone were able to drive B cells to proliferate weakly. In all cases the B cell proliferation induced by gp39-expressing COS cells was reduced to background levels by the addition of soluble CD40. Unlike gp39-expressing COS cells, recombinant soluble gp39 was not mitogenic alone and required co-stimulation to drive B cell proliferation. These results suggest that B cells require a second signal besides gp39–CD40 to drive proliferation and that soluble gp39 alone in a non-membrane bound form is able to provide co-stimulatory signals to B cells.

Key words: Bp50/CD20/fusion protein/IgE/type II membrane protein

Introduction

CD40 is a ~50 kDa glycoprotein expressed on the surface of B cells, follicular dendritic cells, normal basal epithelium and some carcinoma and melanoma derived cell lines (Paulie *et al.*, 1985, 1989; Clark and Ledbetter, 1986; Ledbetter *et al.*, 1987; Young *et al.*, 1989). Isolation of a human cDNA encoding CD40 showed that this protein is a type I membrane protein that is significantly related to the members of the nerve growth factor receptor family (Stamenkovic *et al.*, 1989). The role of CD40 in B cell activation is well established. Crosslinking CD40 with anti-CD40 monoclonal antibodies (mAb) induces B cell aggregation via LFA-1 (Gordon *et al.*, 1988; Barrett *et al.*, 1991), increases Ser/Thr (Gordon *et al.*, 1988) and Tyr (Uckun *et al.*, 1991) phosphorylation of a number of intracellular substrates and provides a 'competence' signal that allows B cells to proliferate and undergo class switching when stimulated with the appropriate second signal. For example, anti-CD40 mAb can synergize with PMA (Gordon *et al.*, 1987) or anti-CD20 mAb (Clark and Ledbetter, 1986) to induce B cell proliferation, with IL-4 to induce B cell proliferation (Gordon *et al.*, 1987; Rousset *et al.*, 1991) and IgE secretion (Jabara *et al.*, 1990; Gascan *et al.*, 1991; Rousset *et al.*, 1991; Zhang *et al.*, 1991; Shapira *et al.*, 1992) and with IL-10 and TGF-β to induce IgA secretion by $sIgD^+$ B cells (DeFrance *et al.*, 1992). There is also evidence that CD40-mediated signals are involved in modulating cytokine production by activated B cells (Cairns *et al.*, 1988; Clark and Shu, 1990). Crosslinking of anti-CD40 mAb alone is not sufficient to induce B cell proliferation as demonstrated by the observation that anti-CD40 mAb immobilized on plastic in conjunction with IL-4 are unable to induce vigorous B cell proliferation (Banchereau *et al.*, 1991). However, anti-CD40 mAb immobilized on murine L cells transfected with an Fc receptor, CD32, are able to induce B cell proliferation in the presence of IL-4 (Banchereau *et al.*, 1991), suggesting that a signal provided by the fibroblasts synergizes with the CD40 signal and IL-4 to drive B cell proliferation.

We and others have used soluble forms of the extracellular domain of human CD40, CD40–Ig, to show that the CD40 ligand gp39 is a glycoprotein of ~39 kDa expressed on the surface of activated $CD4^+$ murine T cells (Armitage *et al.*, 1992; Noelle *et al.*, 1992). Interaction with gp39 induces B cells to enter the cell cycle and become responsive to the growth and differentiation effects of lymphokines (Armitage *et al.*, 1992; Noelle *et al.*, 1992). Both CD40–Ig and a hamster mAb specific to murine gp39, MR1, are able to block B cell proliferation induced by activated T cells (PM^{Act}), but not by lipopolysaccharide (LPS) (Noelle *et al.*, 1992). In addition, the polyclonal differentiation of B cells to produce Ig elicited by T cells in conjunction with IL-4 and IL-5 can be blocked by both CD40–Ig and MR1 (Noelle *et al.*, 1992). Recently, a cDNA encoding murine gp39 has been isolated and shown to be functionally active when expressed as a membrane protein on transfected cells (Armitage *et al.*, 1992). This cDNA encodes a 260 amino acid polypeptide with the typical features of a type II membrane protein and CV1/EBNA cells expressing murine gp39 were shown to induce murine and human B cell proliferation without additional co-stimulus.

Here we report the isolation and characterization of a human cDNA encoding gp39, discuss the homology of gp39 with tumor necrosis factor (TNF) and describe the preparation of a functional, soluble and recombinant form of gp39. Using the soluble recombinant gp39 we show that human gp39 is unable to induce the proliferation of B cells in the

absence of a co-stimulus but is able to synergize with known B cell co-stimuli.

Results

Isolation and characterization of a cDNA encoding human gp39

A cDNA encoding the human gp39 was amplified from a cDNA library prepared from mRNA isolated from PHA activated human peripheral blood T cells by PCR using synthetic oligonucleotides based on the murine gp39 sequence (Armitage *et al.*, 1992). The PCR product was subcloned into the expression vector CDM8 (Seed, 1987). COS cells transfected with the CDM8–gp39 plasmid produced a cell surface protein that bound to CD40–Ig (data not shown) (Noelle *et al.*, 1992). A complete human gp39 gene was isolated by colony hybridization from the same cDNA library that was used for the PCR amplification of gp39 using the subcloned PCR product as a probe. A number of positive clones were isolated and analyzed by restriction enzyme digestion. DNA corresponding to those clones containing the largest inserts, 1.8–1.5 kb, were transfected into COS cells and their ability to direct the expression of a CD40–Ig binding protein examined. One such clone that was positive by these criteria was analyzed further and is referred to hereafter as human gp39. Immunoprecipitation of cDNA-encoded human gp39 protein from transfected COS cells using CD40–Ig showed a single band corresponding to a molecular mass of ~32–33 kDa (Figure 1). The COS cell derived protein is smaller than expected based on previous studies of murine gp39. However, we have observed in many instances that the apparent molecular masses of a number of different T cell surface proteins obtained from COS cell transfectants are smaller than those obtained from T cells (Aruffo and Seed, 1987; Aruffo *et al.*, 1991). We believe that these differences in size are the result of incomplete glycosylation of the proteins by COS cells.

The isolated human gp39 clone is ~1.8 kb, encoding a polypeptide of 261 amino acids (aa) with a predicted molecular mass of ~29 kDa. The absence of a secretory signal peptide suggests that the first 22 amino-terminal residues are intracellular. This is followed by 24 predominantly hydrophobic residues consistent with a transmembrane domain and 215 aa constituting the putative carboxy-terminal extracellular (EC) domain. One N-linked glycosylation site (Asn-X-Ser/Thr) exists in the EC and one in the cytoplasmic domain (nucleotide sequences corresponding to coding sequence and the predicted aa sequence are shown in Figure 2A). The expected orientation of the protein, with an extracellular carboxy-terminus, classifies it as a type II membrane protein and the difference between the predicted and observed molecular mass suggests that it undergoes post-translational modifications, most probably the addition of carbohydrate groups. Murine (Armitage *et al.*, 1992) and human gp39 are highly homologous. Within the coding region they share ~83% identity at the nucleotide level and ~73% identity at the aa level (Figure 2B; data not shown). The predicted amino acid sequence of human gp39 was compared with those in the National Biomedical Research Foundation (NBRF) database using the FASTP algorithm and found to have significant homology with TNFα (Gray *et al.*, 1984) and TNFβ (Pennica *et al.*, 1984; Wang *et al.*, 1985) (Figure 2B).

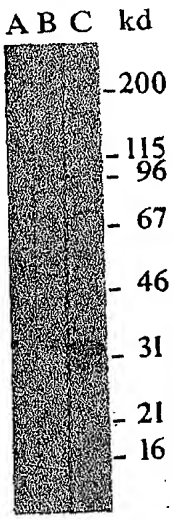

Fig. 1. Precipitation of gp39 from COS cells using CD40–Ig. Radiolabeled proteins from surface iodinated COS cells transfected with vector alone (mock, lane A) or gp39 (lanes B–C) were precipitated with CD40–Ig (lanes A and C) or Leu8–Ig (lane B) and analyzed by SDS–PAGE under reducing conditions as described in Materials and methods. The electrophoretic mobility of molecular mass standards of the indicated mass in kDa are shown to the right.

Preparation and characterization of the soluble recombinant gp39 as a chimeric fusion protein

Previously, recombinant soluble forms of type I cell surface proteins have been generated as fusion proteins in which the EC domain (amino-terminal domain) of the membrane proteins have been joined to a tag polypeptide, such as an immunoglobulin heavy chain constant region (e.g. CD40–Ig and Leu8–Ig fusions used in this study) (for a review see Hollenbaugh *et al.*, 1992). In these cases, the Ig region replaces the transmembrane and cytoplasmic domains of the surface proteins and is used as a 'handle' for manipulating the soluble recombinant surface proteins with reagents developed for use with antibodies. Because type II membrane proteins are oriented with a carboxy-terminal EC domain, an alternate fusion was designed such that the tag polypeptide is amino-terminal to the EC portion of the protein, again replacing the transmembrane and cytoplasmic domains of the surface protein. This tag polypeptide should also contain an amino-terminal secretory signal sequence to allow export of the fusion protein. We chose the murine CD8 EC domain (Nakauchi *et al.*, 1985) as our tag polypeptide to construct our fusion proteins of type II membrane proteins for four reasons: (i) the use of a complete extracellular protein domain as the tag polypeptide minimizes the chances that the tag polypeptide will affect the tertiary structure of the surface protein to which it is fused while maximizing the likelihood that the fusion protein will be expressed and exported; (ii) a previously studied CD8–Ig chimera demonstrated that CD8 fusion proteins are produced and exported by COS cells in high yield; (iii) a large number of mAb directed to CD8 are available and can be used to manipulate the recombinant CD8 fusion proteins; and (iv) the interaction between murine CD8 and human MHC I is not detectable. To generate the CD8–gp39 fusion gene, $_s$gp39, a cDNA fragment encoding the EC domain of murine CD8, was fused with a cDNA fragment encoding the EC domain of gp39, as described in Materials and methods (Figure 3A). The $_s$gp39

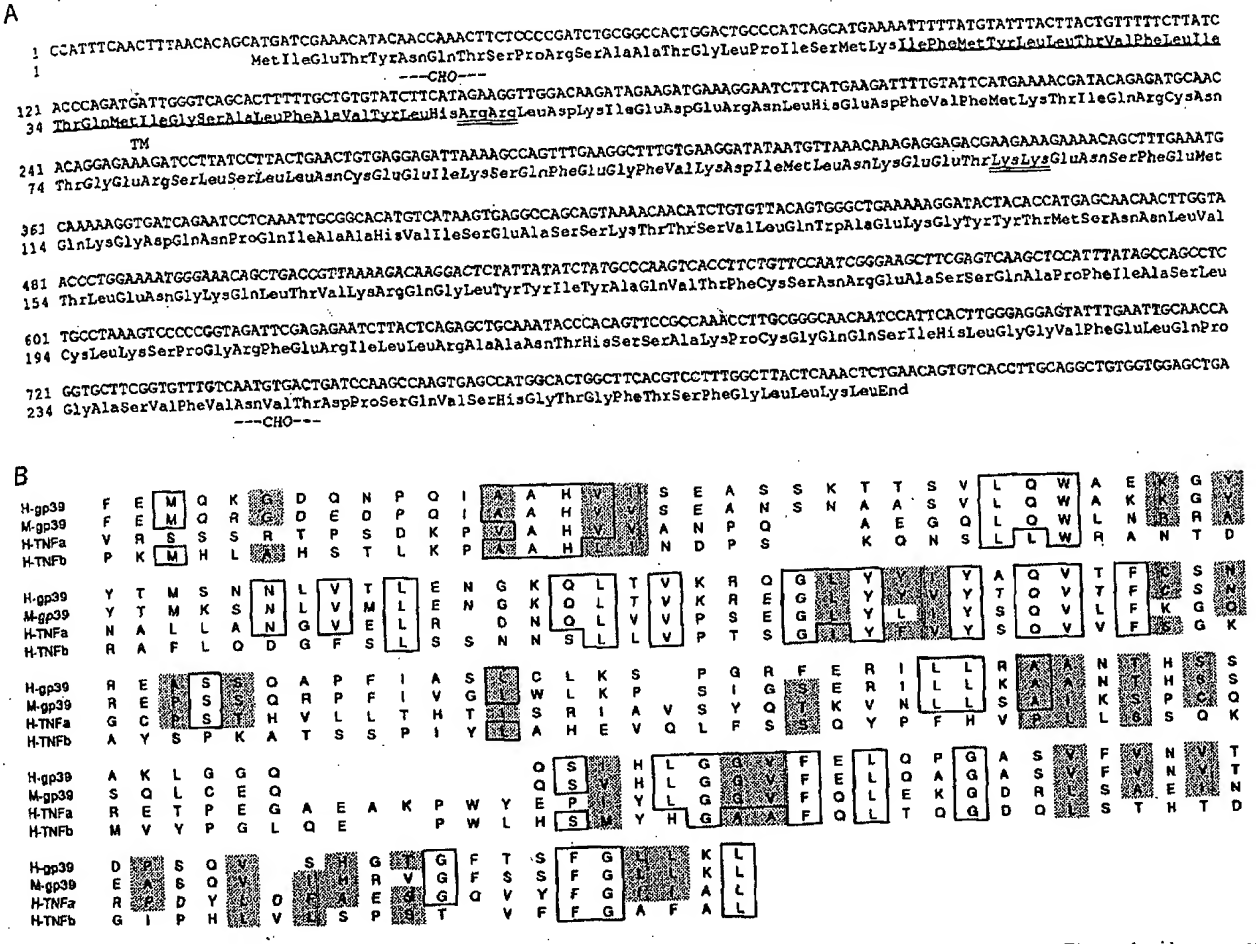

Fig. 2. Nucleotide and predicted amino acid sequence of human gp39 and homology to murine gp39, TNFα and TNFβ. (A) The nucleotide sequence and translated ORF are numbered at left. Sites of potential N-linked glycosylation are marked (CHO), the predicted transmembrane domain (TM) is underlined and the two Arg residues and the two Lys residues discussed in the text are double underlined. Nucleotide and amino acid numbering is given to the left. The nucleotide sequence is available from EMBL/GenBank/DDBJ under accession number Z15017. (B) Alignment of the predicted amino acid sequence of human gp39 (H-gp39), murine gp39 (M-gp39), human TNFα (H-TNFα), and human TNFβ (H-TNFβ). Amino acids shared by at least three proteins are shown boxed; similar amino acids shared by at least three of the proteins are shown shaded.

protein was prepared by transient expression in COS cells and recovered from COS cell supernatants with anti-CD8 mAb or with a soluble recombinant CD40–Ig chimera, which we used in our earlier murine gp39 studies (Figure 3B). The $_s$gp39 protein has a molecular mass of ~50 kDa (Figure 3B) when analyzed by SDS–PAGE under reducing conditions. Preliminary results indicate that $_s$gp39 forms dimers and trimers in solution (data not shown).

As a control, a chimeric gene encoding a soluble recombinant form of the B cell antigen CD72 (Von Heogen *et al.*, 1990), another type II membrane protein, was constructed (Figure 3A). The $_s$CD72 protein was also produced by transient expression in COS cells and recovered from COS cell supernatants with anti-CD8 mAb or with three anti-CD72 mAb tested, but not with the CD40–Ig fusion protein (Figure 3C; data not shown).

To characterize further the interaction between CD40 and the soluble recombinant gp39, COS cells were transfected with a cDNA encoding the full length CD40 protein (Stamenkovic *et al.*, 1989) and their ability to bind to $_s$gp39, $_s$CD72 and anti-CD40 mAb examined by fluorescence microscopy. Both the $_s$gp39 and the anti-CD40 mAb bound to the transfectants while $_s$CD72 did not (Figure 4; data not shown). In addition, COS cells were transfected with a cDNA encoding the full length gp39 and their ability to bind to CD40–Ig (Noelle *et al.*, 1992) or an irrelevant Ig fusion protein, Leu8–Ig (Aruffo *et al.*, 1992), was examined. CD40–Ig, but not Leu8–Ig, bound to gp39-expressing COS cells (Figure 4; data not shown).

In parallel experiments, $_s$gp39 and $_s$CD72 were immobilized in the wells of a 96-well microtiter dish via an anti-CD8 mAb and their binding was examined in relation to increasing concentrations of CD40–Ig or a control immunoglobulin fusion protein, Leu8–Ig. The binding of CD40–Ig to immobilized $_s$gp39 was saturable, while CD40–Ig did not bind to $_s$CD72 and Leu8–Ig did not bind to $_s$gp39 (Figure 5). Similarly, CD40–Ig and Leu8–Ig were immobilized via anti-human Fc antibodies and the binding of $_s$gp39 or $_s$CD72 from increasing dilutions of COS cell supernatants was measured. Immobilized CD40–Ig bound $_s$gp39 but not $_s$CD72 and Leu8–Ig did not bind $_s$gp39 (data not shown).

Human gp39 requires a co-stimulus to induce B cell proliferation

To examine the role of gp39–CD40 interactions in B cell activation, COS cells transfected with either the cDNA

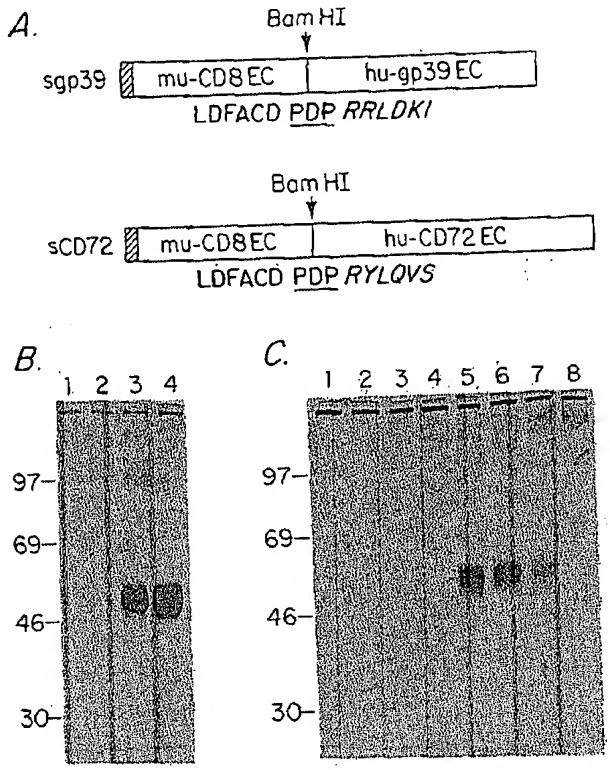

Fig. 3. Soluble recombinant human gp39 and CD72, $_s$gp39 and $_s$CD72. (**A**) The cDNA fragment encoding the extracellular domain of murine CD8 is designated, mu-CD8 EC. The murine CD8 amino terminal secretory signal sequence is shown stippled. The cDNA fragments encoding the extracellular domain of human gp39 or CD72 are designated hu-gp39 EC and hu-CD72 EC, respectively. The amino acid sequences predicted at the site of fusion of the extracellular domain of murine CD8 and human gp39 (italic) or CD72 (italic) are shown below the individual diagrams. Residues introduced at the junction of the two cDNA fragments are shown underlined. The unique *Bam*HI restriction enzyme recognition site at the junction of the two genes is shown. (**B**) Radiolabeled proteins from the supernatants of metabolically-labeled mock (lanes 1 and 2) or CD8–gp39 (lanes 3 and 4) transfected COS cells were immunoprecipitated based on their interaction with the anti-murine CD8 mAb 53-6 (lanes 1 and 3) or the CD40–Ig (lanes 2 and 4) and analyzed by SDS–PAGE under reducing conditions as described in Materials and methods. The electrophoretic mobility of molecular mass standards of the indicated mass in kDa are shown to the left. (**C**) Radiolabeled proteins from the supernatants of metabolically labeled mock (lanes 1–4) and CD8–CD72 (lanes 5–8) transfected COS cells were recovered based on their reactivity with the anti-murine mAb 53-6 (lanes 1 and 5), the anti-CD72 mAb J3101 (lanes 2 and 6), the anti-CD72 mAb BU41 (lanes 3 and 7) and CD40–Ig (lanes 4 and 8) and analyzed by SDS–PAGE under reducing conditions as described in Materials and methods. The electrophoretic mobility of molecular mass standards of the indicated mass in kDa are shown to the left.

encoding gp39 or vector alone (mock) were tested for their ability to stimulate B cell proliferation. Peripheral blood B cells proliferated only weakly when incubated with gp39-expressing COS cells alone (Figure 6). However, upon exposure to gp39-expressing COS cells in conjunction with either the IF5 mAb (Clark *et al.*, 1985) directed against the B cell surface protein CD20 or PMA, vigorous B cell proliferation was observed. In both cases, the gp39-driven B cell proliferation could be reduced to background levels with the soluble CD40–Ig fusion protein (Figure 6). B cells proliferated weakly when incubated with mock-transfected COS cells in the presence of either the anti-CD20 mAb or PMA and this proliferation was unaffected by the presence of CD40–Ig (Figure 6). The weak B cell proliferation observed with gp39-expressing COS cells in the absence of a co-stimulatory signal suggests that in this case COS cells also provide co-stimulatory signals that synergize with CD40 signals to drive B cell proliferation.

Human peripheral blood B cells were incubated with a soluble recombinant gp39, $_s$gp39 or a control fusion protein, $_s$CD72, in the absence or presence of anti-CD20 mAb or PMA. B cells responded with weak proliferation in the presence of $_s$gp39 alone. However, $_s$gp39 induced vigorous B cell proliferation when either anti-CD20 mAb or PMA was present (Figure 7). B cell proliferation was not observed with $_s$CD72, anti-CD20 mAb or PMA alone or with $_s$CD72 in conjunction with anti-CD20 mAb or PMA (Figure 7).

In parallel experiments, dense human tonsillar B cells were prepared and their ability to proliferate in response to $_s$gp39 and $_s$CD72 was examined (Figure 8; data not shown). As seen with peripheral blood B cells, tonsillar B cells proliferated weakly in response to $_s$gp39, but showed strong proliferation when incubated with $_s$gp39 in the presence of the anti-CD20 mAb 1F5 or PMA. No significant proliferation over background levels was observed when the cells were incubated with $_s$CD72 alone or in the presence of the 1F5 mAb or PMA. To examine the specificity of the $_s$gp39-driven activation response, the ability of CD40–Ig to block the $_s$gp39 and 1F5 or $_s$gp39 and PMA driven B cell proliferation was examined. CD40–Ig was able to reduce the $_s$gp39 driven B cell activation under the conditions used (~20 μg/ml gave ~50% inhibition, Figure 8A) while the control fusion protein Leu8–Ig had no effect (Figure 8B).

Discussion

The ability of the B cell surface receptor CD40 to transduce signals has been established using monoclonal antibodies (Clark and Ledbetter, 1986; Gordon *et al.*, 1987). To study further the role of CD40, we have isolated and characterized a cDNA encoding the ligand from a human source. In addition, we have developed a means of generating a soluble form of the protein so that isolated co-stimulatory signals may be studied in the absence of stimuli contributed by the heterologous cells expressing the full length protein.

Isolation of a cDNA clone encoding human gp39 showed that the predicted polypeptide is a type II membrane protein, closely related to its murine homolog and more distantly related to TNFα (Gray *et al.*, 1984) and TNFβ (Pennica *et al.*, 1984; Wang *et al.*, 1985). TNFα and TNFβ are pleiotropic cytokines that exist predominantly as secreted proteins (for a review see Vassalli, 1992), although membrane bound forms have been reported (Kriegler *et al.*, 1988). Presently, there is no evidence for the existence of a soluble form of gp39. However, human gp39, like murine gp39 (Armitage *et al.*, 1992), contains two Arg residues at the junction of the extracellular and transmembrane domains, which may be a site of proteolytic cleavage to give rise to soluble gp39 (Thomas *et al.*, 1988). Alternatively, cleavage could take place at the two Lys residues located at the start of the region of gp39, which is homologous to TNF. The three dimensional structures of TNFα (Eck and Sprang, 1989; Jones *et al.*, 1989) and TNFβ (Eck *et al.*, 1992) have been reported. Soluble TNFα and TNFβ are trimers composed of monomers with a distinctive anti-parallel β-sheet tertiary structure with a 'jelly-roll' topology. Structural

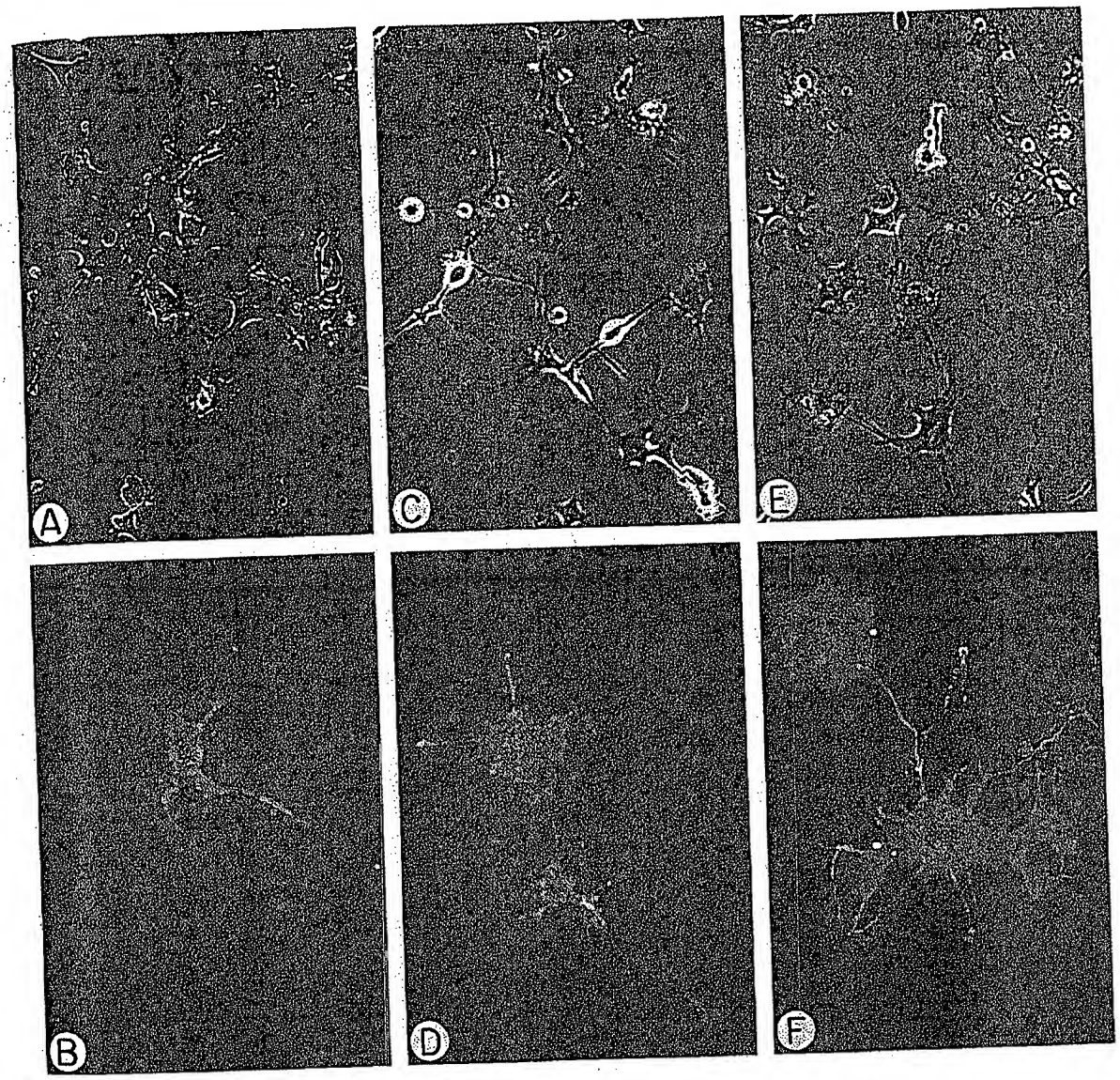

Fig. 4. Binding of $_s$gp39 or CD40-Ig to transfected COS cells. COS cells transfected with either a gp39 (A and B) or a CD40 (C–F) cDNA expression plasmid were examined for their ability to bind either soluble recombinant CD40 (A and B), or soluble recombinant gp39 (C and D) or the anti-CD40 mAb G28-5 (E and F) as described in Materials and methods. Phase (A, C and E) and fluorescence (B, D and F) images of representative fields are shown (magnification, 200×).

alignment studies suggest that the amino acid sequence of gp39 is compatible with a TNF-like fold and the formation of a trimeric quarternary structure (J.Bajorath, personal communication). The proposed relationship between gp39 and TNF is also strengthened by the observation that the gp39 receptor, CD40, is significantly homologous to the members of the TNF receptor superfamily (Stamenkovic *et al.*, 1989) and that similar to gp39, the membrane bound form of TNFα is a type II glycoprotein (Kriegler *et al.*, 1988).

It has been reported that purified murine splenic B cells and human tonsillar B cells proliferate when incubated with CV1/EBNA cells expressing murine gp39 in the absence of co-stimulus (Armitage *et al.*, 1992). Based on these data it had been thought that gp39 is directly mitogenic for B cells. To determine whether gp39 binding to CD40 is able to stimulate B cells to proliferate in the absence of other co-stimulatory signals, the effect of gp39 expressed in fibroblast cells on the proliferation of B cells was compared with that of soluble gp39. The gp39 was active in both forms, however, interesting differences between gp39-expressing COS cells and $_s$gp39 were seen. COS cells expressing human gp39 were able to induce weak B cell proliferation in the absence of co-stimuli and could synergize with either anti-CD20 mAb or PMA to induce vigorous B cell proliferation. In all cases, the B cell proliferation could be reduced to background levels with soluble recombinant gp39 receptor, CD40–Ig. Meanwhile $_s$gp39 was only able to induce B cells, isolated from either peripheral blood or tonsils, to proliferate in conjunction with either anti-CD20 mAb or PMA and as observed with gp39-expressing COS cells, $_s$gp39-driven B cell activation could be inhibited with CD40–Ig, but not with an irrelevant Ig fusion protein. These data indicate that gp39 requires a co-stimulatory signal to drive B cell proliferation most effectively and that there is no strict requirement for cell surface expression of gp39 for activity. In addition, the ability of gp39 expressed on the surface of COS cells to stimulate weak B cell proliferation

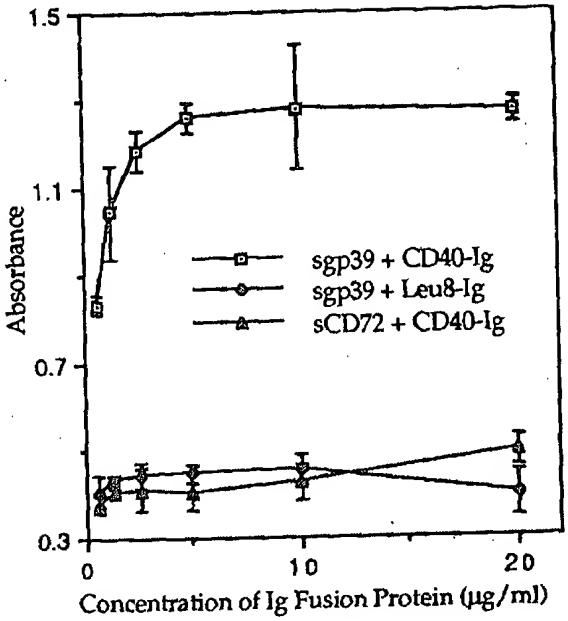

Fig. 5. Characterization of the $_s$gp39–CD40–Ig interaction. The ability of increasing concentrations of CD40–Ig (0.6–20 μg/ml) and the control immunoglobulin fusion protein, Leu8–Ig (0.6–20 μg/ml), to bind to immobilized $_s$gp39 was examined by ELISA as described in Materials and methods. The ability of increasing concentrations of CD40–Ig to bind to the immobilized control fusion protein $_s$CD72 was also examined in the same way. In both cases the $_s$gp39 and $_s$CD72 were immobilized on plastic that had been previously coated with the anti-murine CD8 mAb 53-6 as described in Materials and methods.

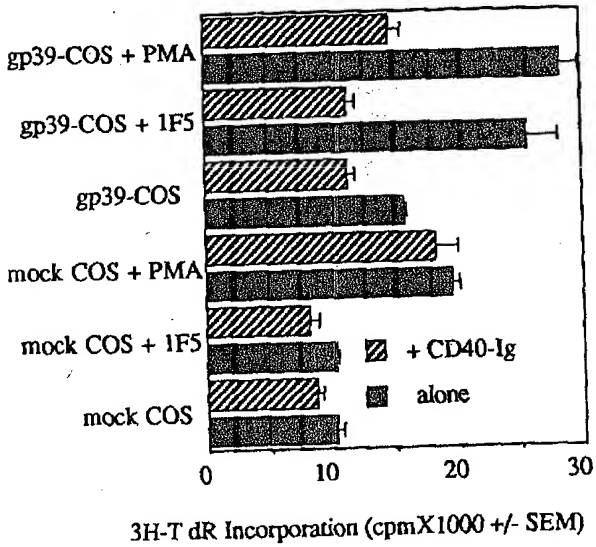

Fig. 6. Activation of human B cells by surface bound gp39. The ability of gp39-expressing COS cells (gp39-COS) or mock transfected COS cells (mock COS) to stimulate the proliferation of human peripheral blood B cells alone or in the presence of the anti-CD20 mAb 1F5 (+ 1F5) or PMA (+ PMA) in the absence (solid bars, alone) or presence of CD40–Ig (hatched bars, + CD40–Ig) was examined as described in Materials and methods and evaluated by [^{3}H]thymidine incorporation.

supports the idea that COS cells may also provide co-stimulatory signals, as yet undefined, which can weakly synergize with those provided by gp39. These observations parallel those in which L cell transfectants expressing the

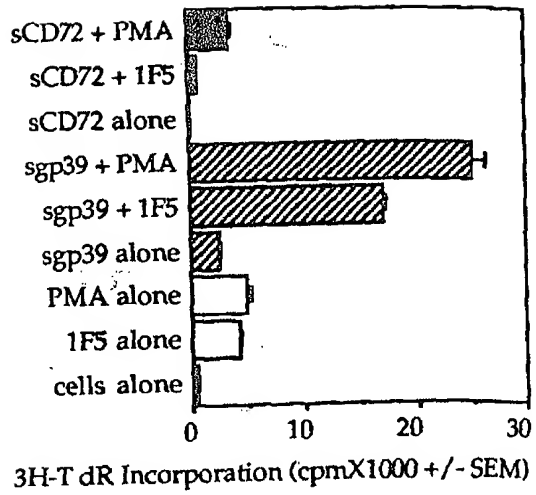

Fig. 7. Activation of human peripheral blood B cells by $_s$gp39. The ability of soluble recombinant gp39 ($_s$gp39, hatched bars) or control soluble recombinant fusion protein ($_s$CD72, solid bars) to stimulate the proliferation of human peripheral blood B cells alone or in conjunction with the anti-CD20 mAb 1F5 (+ 1F5) or PMA (+ PMA) was examined as described in the Materials and methods, evaluated by [^{3}H]thymidine incorporation and compared to that of B cells incubated for an equivalent amount of time in the absence of exogenous stimuli (cells alone, open bars) or in the presence of either 1F5 alone or PMA alone (open bars).

Fc receptor CD32 presenting anti-CD40 mAb in conjunction with IL-4 were found to induce B cells to proliferate more efficiently than did anti-CD40 mAb immobilized on plastic in conjunction with IL-4 (Banchereau *et al.*, 1991). Thus the findings suggest that fibroblasts are capable of providing stimulatory signals that synergize with those provided by anti-CD40 mAb resulting in efficient B cell proliferation.

The development of factor-dependent, long-term B cell cultures has important implications for the study of B cell growth and differentiation, and the development of antigen-specific B cell lines (Tisch *et al.*, 1988). Experiments with anti-CD40 mAb showed that CD40 signals can synergize with other co-stimulatory signals such as those delivered by anti-CD20 mAb to drive B cell proliferation, and that treatment of B cells with anti-CD40 mAb induces a state of B cell 'alertness' that allows them to respond more readily to subsequent activation signals. These observations led to the development of long-term B cell cultures using the L cell/CD32/anti-CD40 mAb system described above. The ability of $_s$gp39 to stimulate B cell proliferation in conjunction with anti-CD20 mAb or PMA suggests that it might replace the anti-CD40 presenting L cells in an *in vitro* system for long-term B cell growth. Further work is needed to investigate whether $_s$gp39 will function as a novel recombinant B cell growth factor that can be used in conjunction with IL-4 or other growth factors in a simple *in vitro* system for the long-term propagation of B cell cultures. It is interesting to note that the CD40–Ig and the $_s$gp39 fusion proteins described here can be used to either inhibit or stimulate the CD40 response in B cells and thus are useful research tools with which to study B cell–T cell interactions and might have a clinical application by permitting either up regulation or down regulation of B cell responses.

Recently, a large number of cDNA clones encoding type II

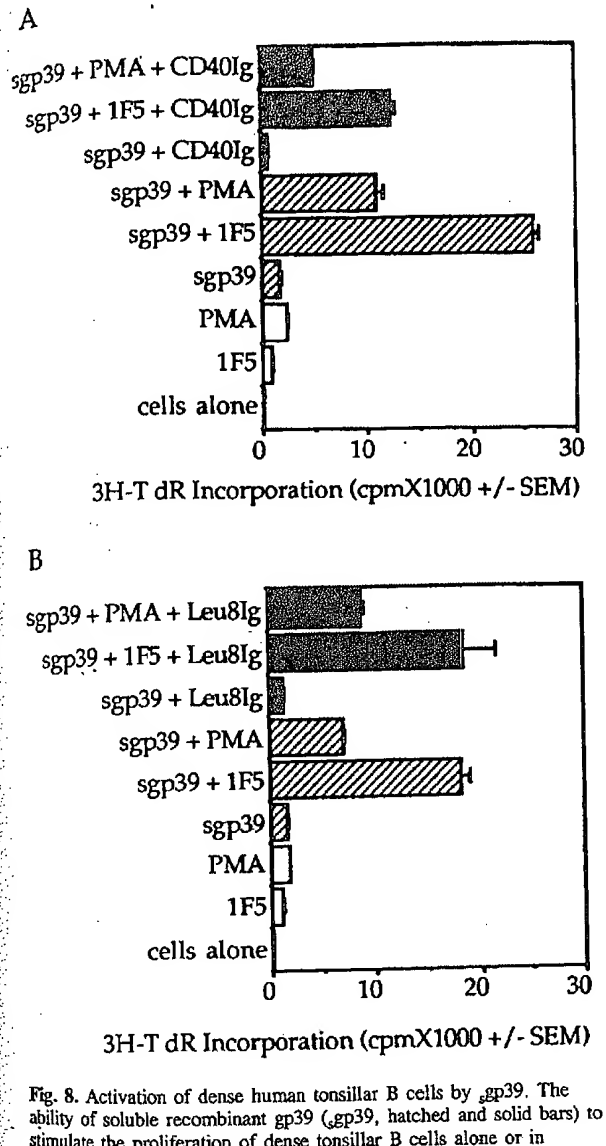

Fig. 8. Activation of dense human tonsillar B cells by $_s$gp39. The ability of soluble recombinant gp39 ($_s$gp39, hatched and solid bars) to stimulate the proliferation of dense tonsillar B cells alone or in conjunction with the anti-CD20 mAb 1F5 (+1F5) or PMA (+ PMA) was examined as described in the Materials and methods, evaluated by [^{3}H]thymidine incorporation and compared to that of B cells incubated alone (cells alone, open bars) or in the presence of either 1F5 alone or PMA alone (open bars). The ability of CD40-Ig (solid bars) to block the $_s$gp39-driven B cell activation was examined at a concentration of 20 μg/ml (A) and compared with an equal concentration of an irrelevant immunoglobulin fusion protein, Leu8-Ig (solid bars, B).

membrane proteins have been isolated. These proteins include amongst others the B cell antigen CD72 (Von Hoegen *et al.*, 1990), which has been reported to interact with the T cell protein CD5 (Van de Velde *et al.*, 1991) and the T cell antigen CD26, a dipeptidyl peptidase (Marguet *et al.*, 1992). Methodologies designed to prepare and purify recombinant soluble forms of these proteins will have a significant impact in facilitating studies on their structure and function. Although recombinant forms of type II membrane proteins have been prepared in the past by replacing the cytoplasmic domain of the type II protein with a cleavable signal peptide (Lemay *et al.*, 1989), these methods have not been used widely since they do not provide for facile purification and manipulation of the recombinant protein unless antibodies directed against it are readily available. Here an alternative method for the preparation of recombinant soluble forms of type II membrane proteins is described in which the extracellular domain of a type I membrane protein serves as a tag polypeptide replacing the transmembrane and cytoplasmic domain of the type II membrane protein. The resulting chimeric protein is secreted into the supernatant of appropriately transfected cells, from which it can be purified and manipulated with antibodies directed to the tag polypeptide. The extensive immunoprecipitation and cell binding studies presented herein for the chimeric soluble forms of gp39 and CD72 suggest that this type of fusion protein is structurally and functionally sound and thus provide a valuable tool with which to study the function of type II membrane proteins *in vitro* and possibly *in vivo*.

Materials and methods

Isolation and characterization of a human cDNA encoding gp39

The previously described CD40-Ig was modified by the introduction of three mutations in the immunoglobulin domain to reduce the binding to Fc receptors (W.Brady and P.Linsley, personal communication). The modified CD40-Ig was purified from COS cell supernatants as previously described by Aruffo *et al.* (1990). Human gp39 cDNA was amplified by PCR from a library prepared from mRNA isolated from PHA-activated human peripheral blood T-cells (Camerini *et al.*, 1989). The oligonucleotide primers were designed based on the sequence of the murine gp39 (Armitage *et al.*, 1992) and included sites for the restriction enzymes *Xba*I and *Hin*dIII to be used in subcloning the PCR product. The oligonucleotides used were: 5′-GCG AAG CTT TCA GTC AGC ATG ATA GAA ACA-3′ and 5′-CGC TCT AGA TGT TCA GAG TTT GAG TAA GCC-3′. Amplification was performed with *Taq* polymerase and the reaction buffer recommended by the manufacturer (Perkin-Elmer Cetus) using 30 cycles of the following temperature program: 2 min at 95°C, 2 min at 55°C and 3 min at 72°C. The PCR product was digested with *Hin*dIII and *Xba*I, and was found to contain an internal *Hin*dIII restriction site. The *Hin*dIII-*Xba*I fragment was subcloned into the CDM8 vector. The complete gene product was constructed by subcloning the *Hin*dIII-*Hin*dIII fragment into the vector containing the *Hin*dIII-*Xba*I fragment. The resulting construct was transfected into COS cells using DEAE-dextran as previously described by Aruffo *et al.* (1990). Transfectants were stained with CD40-Ig (25 μg/ml in DMEM) followed by FITC-conjugated goat anti-human IgG Fc antibody (1:50 dilution in DMEM; TAGO, Burlingame, CA) and visualized by immunofluorescence microscopy. The complete human gp39 was obtained by colony hybridization as described (Sambrook *et al.*, 1989). The subcloned *Hin*dIII-*Hin*dIII fragment of the PCR product was used to generate a ^{32}P-labeled probe by random primed polymerization. Plasmid DNA from three individual clones was transfected into COS cells and cells were stained with CD40-Ig. One clone, clone 19, was positive by this criteria and used in the remainder of the study. The sequence was determined by dideoxy sequencing using Sequenase™ (United States Biochemical Co, Cleveland, OH).

Plasmid containing gp39 or vector alone (mock) was transfected into COS cells by the DEAE-dextran method. 48 h post-transfection, cells were detached with EDTA, radiolabeled with ^{125}I using Iodo-beads (Pierce Chemical Co., Rockford, IL) and lysed with 1% NP-40 with aprotinin as protease inhibitor. Lysates were incubated with 10 μg of CD40-Ig or Leu8-Ig at 4°C overnight in the presence of Protein A-Sepharose beads (Pierce Chemical Co.). Beads were washed several times in lysis buffer and precipitated proteins eluted by boiling in loading buffer in the presence of 2% β-mercaptoethanol. Samples were subjected to SDS-8%PAGE, the gel was dried and exposed for autoradiography at −80°C overnight.

Construction, characterization and preparation of a soluble gp39 chimera

The extracellular domain of the human gp39 was amplified from the cDNA library prepared from mRNA from PHA activated human peripheral blood lymphocytes. The oligonucleotide primers were designed based on sequence information obtained from the PCR product described above and were designed to place a *Bam*HI site at the 5′ end of the gene such that the reading frame would be preserved when the chimeric gene was constructed. The oligonucleotides used were 5′-CGA AGC TTG GAT CCG AGG AGG TTG GAC AAG ATA GAA GAT-3′ and 5′-CGC TCT AGA TGT TCA GAG TTT GAG TAA GCC-3′. The PCR was performed using the *Pfu* polymerase

with the buffer supplied by the manufacturer (Stratagene, La Jolla, CA) with the following temperature program: 5 min at 95°C, 2 min at 72°C and 2 min at 55°C; 40 cycles of amplification consisting of 1 min at 95°C, 2 min at 55°C, 3 min at 72°C and 10 min at 72°C. The PCR product was digested with *Bam*HI and *Xba*I, and subcloned in a vector containing the gene encoding the murine CD8 (Lyt2a) extracellular domain with a *Bam*HI restriction site generated by PCR (N.J.Chalupny, unpublished data). Similarly, the gene encoding the extracellular domain of human CD72 was generated by PCR to contain a *Bam*HI restriction site and subcloned in the CD8-containing vector in the same manner using the oligonucleotides 5′-CGA AGC TTG GAT CCG CGC TAT CTG CAG GTG TCT CAG CAG-3′ and 5′-CGC TCT AGA CTC GAG GTC CTA ATC TGG AAA CCT GAA AGC-3′ and *Taq* polymerase (Perkin–Elmer Cetus).

The ability of COS cells to express and export $_s$gp39 *and* $_s$CD72 *was* tested. First, COS cells were transfected using DEAE-dextran. One day after transfection, cells were trypsinized and replated. One day later, cells were fixed with 2% formaldehyde in PBS (20 min at room temperature) and permeabilized with 2% formaldehyde in PBS containing 0.1% Triton X-100 (20 min at room temperature). Cells transfected with $_s$gp39 were stained with CD40–Ig (25 μg/ml in DMEM for 30 min at room temperature) followed by FITC-conjugated goat anti-human Fc antibody (TAGO, Burlingame, CA) diluted 1:500 in DMEM. Cells transfected with $_s$CD72 were stained with the anti-CD72 antibody BU40 (The Binding Site, Birmingham, UK) followed by FITC-conjugated goat anti-mouse Fc antibody (TAGO, Burlingame, CA) diluted 1:500 in DMEM.

COS cells transfected with the $_s$gp39 or $_s$CD72 constructs or vector alone (mock) were grown overnight in Cys- and Met-free DMEM to which [^{35}S]L-methionine and [^{35}S]L-cysteine had been added (Tran^{35}S-label, ICN, Costa Mesa, CA, 27 μCi/ml). *Supernatants were harvested and centrifuged* at 1000 r.p.m. for 10 min. Fusion proteins were recovered from the supernatant using CD40–Ig, 53-6 (anti-murine CD8) plus goat anti-rat Fc, BU40, BU41 (The Binding Site, Birmingham, UK) plus goat anti-mouse IgM Fc or J3:101 (AMAC Inc., Westbrook, ME). Goat antibodies were purchased from Organon Teknika Co., West Chester, PA. For each sample, 1 ml of supernatant, 75 μl Protein A–Sepharose (Repligen, Cambridge, MA) and the precipitating agent(s) were mixed and incubated at 4°C for 2 h. The Sepharose was washed extensively with PBS containing 0.1% NP-40 and resuspended in loading buffer containing 5% β-mercaptoethanol. Proteins were subjected to SDS–PAGE in an 8% polyacrylamide gel. The gel was fixed, dried and exposed to film. COS cell supernatants containing $_s$gp39 or $_s$CD72 were generated by transfection of COS cells. One day after transfection, cell medium was changed to DMEM containing 2% FBS. Supernatants were harvested 8 days after transfection.

Binding assays

The binding of gp39 and CD40 to the soluble forms of their respective ligands was tested by staining of transfected COS cells. COS cells were transfected with CD40, gp39 or vector alone (mock) using DEAE-dextran. One day after transfection, cells were trypsinized and replated. Cells were stained on the following day. Cells expressing gp39 or mock transfected cells were stained with CD40–Ig (25 μg/ml) followed by FITC-conjugated goat anti-human Fc. Cells expressing CD40 were stained by incubation with COS cell supernatants containing $_s$gp39 followed by mAb 53-6 (anti-murine CD8, 2.5 μg/ml) then FITC-conjugated goat anti-rat Fc (Organon Teknika Co., West Chester, PA, 1.5 μg/ml). As controls, COS cells expressing CD40 were stained with FITC-conjugated G28-5 (anti-CD40) or using COS cell supernatants containing $_s$CD72. All incubations were done at room temperature in PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 2% FBS *and the same buffer was used for all washes. Following staining,* cells were fixed with 1% paraformaldehyde in PBS.

The binding of $_s$gp39 to CD40–Ig was investigated by ELISA. Wells of a 96-well plate (Immunolon-2, Dynatech) were coated with 53-6 antibody (anti-murine CD8, 10 μg/ml, 100 μl/well and 50 mM sodium bicarbonate, pH 9.6 for 1 h at room temperature). Wells were washed with phosphate buffered saline containing 0.05% Tween-20 (TPBS) and blocked with 1 × specimen diluent concentrate (Genetic Systems, Seattle, WA, 225 μl/well for 2 h at room temperature). Wells were washed with TPBS. Supernatants from COS cells expressing either $_s$gp39 or $_s$CD72 were added (150 μl/well) and plates were incubated at 4°C overnight. Wells were washed (TPBS) and fusion proteins CD40–Ig or Leu8–Ig were added (serially diluted in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, 20–0.6 μg/ml, 100 μl/well for 1 h at room temperature). Wells were washed in TPBS and peroxidase-conjugated goat F(ab′)2 anti-human IgG was added to each well (TAGO, *Burlingame, CA, 1:5000 dilution in 1 × specimen diluent,* 100 μl/well for 1 h at room temperature). Wells were washed in TPBS and chromogenic substrate was added (Genetic Systems chromagen diluted 1:100 in EIA Buffered Substrate, Genetic Systems, 100 μl/well). The reaction was stopped after 10 min with the addition of Stop Buffer (Genetic Systems, 100 μl/well) and the absorbance was measured on an ELISA reader at dual wavelengths, 450 and 630 nm. Additionally, the ELISA was performed by immobilization of CD40–Ig on plates coated with goat anti-human Fc. Binding of $_s$gp39 from increasing dilutions of COS cell supernatants was detected using 53-6 mAb followed by FITC conjugated goat anti-rat Fc. Fluorescence was measured on a microplate reader.

B cell proliferation assays

PBMC were isolated by centrifugation through lymphocyte separation medium (Litton Bionetics, Kensington, MD). Human B lymphocytes were enriched from PBMC by passage of cells over nylon columns (Wako Chemicals USA, Inc., Richmond, VA) and harvesting of adherent cells. These cells were then treated with leu–leu methyl ester (Sigma, St Louis, MO) to deplete monocytes and NK cells. The resulting cell population was analyzed by flow cytometry on an EPICS C (Coulter Electronics, Hialeah, FL) and consisted of 50% human peripheral B cells.

Tonsillar B cells were prepared from intact tonsils by mincing to give a tonsillar cell suspension. The cells were then centrifuged through Lymphocyte Separation Medium, washed twice and fractionated on a discontinuous Percoll (Sigma, St Louis, MO) gradient. Cells with a density >50% were collected, washed twice and used in proliferation assays.

COS cells transfected with the gp39 construct or vector alone (mock-COS) were harvested from tissue culture plates with EDTA, washed twice with PBS, suspended at 5 × 10^6 cells/ml and irradiated with 5000 rads from a ^{137}Cs source. COS cells were used at a ratio of 1:4 (1 × 10^4 COS cells: 4 × 10^4 B cells) in proliferation assays.

Measurement of proliferation was performed by culturing cells in quadruplicate samples in flat-bottomed 96-well microtiter plates at 5 × 10^4 cells/well in complete RPMI medium containing 10% FCS. Reagents used were 1F5 (anti-CD20, 1 μg/ml), PMA (10 ng/ml, LC Services Woburn, MA), G28-5 (anti-CD40, 1 μg/ml), CD40–Ig (5 μg/ml *in assays of peripheral blood B cells, 20 μg/ml in assays of tonsillar B cells*) and supernatants of COS cells expressing $_s$gp39 or $_s$CD72 (diluted 1:4). Cell proliferation was measured by uptake of [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) after 5 days of culture and an overnight pulse. Cells were harvested onto glass fibre filters and radioactivity was measured in a liquid scintillation counter.

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While this manuscript was in press, sequence similarity between the murine ligand of CD40 and TNF-a and TNF-b was described [*Nature*, **358**, 26 (1992)].